The University of Manitoba

TOLEROGENIC MONOMETHOXYPOLYETHYLENE GLYCOL DERIVATIVES OF XENOGENEIC MONOCLONAL IMMUNOGLOBULINS

by

Ian Wilkinson

A thesis

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DOCTOR OF PHILOSOPHY

To Mam, Dad and Sandra

"Just the place for a Snark!" the Bellman cried,
As he landed his crew with care;
Supporting each man on the top of the tide
By a finger entwined in his hair

"Just the place for a Snark! I have said it twice:
That alone should encourage the crew.
Just the place for a Snark! - I have said it thrice:
What I tell you three times is true."

Lewis Carroll, "The Hunting of the Snark"

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Abstract

Monoclonal antibodies (MAbs) specific for tumor antigens ("magic bullets") could be used to specifically eliminate malignant cells. However, the administration of xenogeneic immunoglobulins (XIgG) often elicits host anti-XIgG antibodies. The work presented here demonstrates a possible means of abrogating this response, using monomethoxypolyethylene glycol derivatives of XIgG MAbs, in a mouse model.

It has been demonstrated that monomethoxypolyethylene glycol (mPEG) derivatives of human myeloma IgG ($HIgG(mPEG)_n$, where n = 5,13,17 or 20) at doses of 10 or 100 micrograms, can induce specific suppression of the IgG immune response to heat-aggregated human myeloma IgG in female BDF1 mice. The amount of suppression induced was related both to the dose of conjugate administered and to the degree of substitution, n.

In vivo studies showed that $HIgG(mPEG)_{20}$ remained in circulation at levels six fold greater than those of HIgG subjected to sham conjugation with hydrolysed (inactivated) mPEG (sham conjugated HIgG) and that there was no accumulation of conjugate in organs.

Studies on the kinetics of tolerance induction by 100 micrograms of $HIgG(mPEG)_{20}$ showed that conjugate could induce tolerance if it were administered from 6 to 43 days prior to immunisation. Administration one day before immunisation also resulted in suppression but to a lesser degree. Administration of conjugate immediately before or 6 hrs or 63 days before immunisation had no

significant effect. In contrast enhancement of the immune response was observed when $HIgG(mPEG)_{20}$ was administered 2 days after immunisation.

It was shown that multiple injections of 100 micrograms of $HIgG(mPEG)_{17}$ could suppress the immune response to 14 immunising injections of heat aggregated human IgG (haIgG).

The specificity of the tolerance induced by $HIgG(mPEG)_{17}$ was found to extend to the IgG human myeloma immunoglobulin (HIgG#1) and to bovine IgG (BIgG) but not to ovalbumin (OA).

The mechanism of tolerance was investigated. Specific tolerance could be transferred into naive syngeneic recipients by unfractionated splenic leukocytes derived from tolerised donor mice; surface immunoglobulin negative (sIg-); surface immunoglobulin negative/receptor for human IgG positive (sIG-/rHIgG+); surface immunoglobulin negative/receptor for human IgG negative (sIg-/rHIgG-) splenic leukocytes and freeze/thaw (F/T) extracts of sIg- splenic leukocytes, but not by surface immunoglobulin positive (sIg+) leukocytes. The suppressive potency of these cells could be ranked as follows: sIg-/rHIgG+ > sIg- > sIg-/rHIgG- > unfractionated leukocytes. Transfer of tolerance was not due to carry-over of tolerogen as the amount of tolerogen transferred was 100-1000 fold less than that required to induce tolerance.

The cell population responsible for transferable tolerance was shown to be Lyt-2.2 positive.

A difference in kinetics was observed between transferable

tolerance and tolerance in the intact mouse suggesting that T suppressor cells might not be relevant to the maintenance of tolerance in the intact mouse.

Some biophysical characteristics of the suppressive freeze/thaw extracts (designated F/T_{20} and F/T_{17}) were studied. The suppressive factor(s) present in the F/T extracts had a molecular weight range of 13.7 to 25 kD.

The suppressive factor(s) present in F/T_{20} were at least in part composed of protein since the suppressive capacity of the extract could be reduced by incubating the extract with either soluble or insoluble Protease before injection into naive syngeneic recipients . Incubation of the extracts with RNAse or with DNAse did not reduce suppressive capacity.

The suppression induced by the suppressive factor(s) present in F/T_{17} could be reduced by incubation of the extract with HIgG#1, or a different human IgG myeloma (HIgG#2) or BIgG but not by incubation with anti-sera to any of these three immunoglobulins. This implies that (a) the factor(s) recognise some common determinant(s) present on the immunoglobulins; (b) intact HIgG from the $HIgG(mPEG)_{17}$ conjugate used to tolerise the donor mice was not associated with the suppressive factor(s) present in F/T_{17}.

F/T_{17} was unable to transfer suppression between mouse strains differing with respect to the major histocompatibility complex (MHC) i.e., F/T_{17} derived from BDF1 (C57BL/6 x DBA/2) mice (haplotype: b/d) could induce suppression into either parental strain i.e.,

C57BL/6 (haplotype: b) or DBA/2 (haplotype: d), but not into C3H (haplotype: k). All strains could be tolerised by direct administration of HIgG(mPEG)$_{17}$ although to differing degrees.

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List of abbreviations

AOA	aggregated ovalbumin
APC	antigen presenting cell
BCGF	B cell growth factor
BGG	bovine gamma globulin
BIgG	bovine IgG
bps	bytes per second
BSA	bovine serum albumin
C	complement
C.I.	cytotoxic index
CLL	chronic lymphocytic leukemia
CTCL	cutaneous T cell lymphoma
cpm	counts per minute
Da	Daltons
DNP	dinitrophenol
ELISA	enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
FcR	Fc receptor
F/T	Freeze/thaw extract
F/T_{17}	F/T extract of $HIgG(mPEG)_{17}$ tolerised sIg- cells
F/T_{20}	F/T extract of $HIgG(mPEG)_{17}$ tolerised sIg- cells
F/T^{normal}	F/T extract of normal sIg^{-}_{20} cells
FCS	fetal calf serum
haBIgG	heat aggregated bovine IgG
HBs	Hepatitis B surface antigen
haIgG	heat aggregated HIgG
HGG	human gamma globulin
HIgG	human IgG
HIgG#1	human IgG myeloma number 1
HIgG#2	human IgG myeloma number 2
hrs	hours
IL-1	interleukin 1
IL-2	interleukin 2
IL-3	interleukin 3
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
kD	kilo Daltons
maBIgG	mouse anti-bovine IgG
maHIgG#1	mouse anti-human IgG#1
maHIgG#2	mouse anti-human IgG#2
MAb(s)	monoclonal antibody (ies)
MEM	minimal essential medium
MHC	major histocompatibility complex
min	minutes
MLR	mixed lymphocyte reaction
mPEG	monomethoxypolyethylene glycol
OA	ovalbumin

OD	optical density
PBS	phosphate buffered saline
rpm	revolutions per minute
RT	room temperature
S.D.	standard deviation
TRF	T cell replacing factor
XIgG	Xenogeneic IgG

Scope of the present investigation

The aims of this study were to test the hypothesis that HIgG(mPEG)$_n$ conjugates could induce specific suppression of the immune response to the native antigen in a murine model and to study some aspects of the mechanism of this suppression.

In chapter 1 materials and methods are described which are referred to throughout the thesis. The application of XIgG(mPEG)$_n$ conjugates is discussed and the field of tolerance is reviewed with emphasis upon tolerance induced by gamma globulins and protein-mPEG conjugates.

Results of the present study are presented in chapters 2-4. Each group of experiments is described using the following format: introduction, materials and method, results and discussion.

In chapter 2, the synthesis, purification and in vivo testing of XIgG(mPEG)$_n$ conjugates is described including specificity, half-life, and kinetics studies.

In chapter 3 studies on the transfer of tolerance by tolerant unfractionated splenic leukocytes, sIg- , sIG-/rHIgG+, sIg-/rHIgG- splenic leukocytes are described. The relevance of suppressor cells to the maintenance of the tolerance induced by XIgG(mPEG)$_n$ conjugates is investigated through studies of the kinetics of transferable tolerance compared with those of tolerance in the intact animal, and testing for the presence of memory suppressor cells.

In chapter 4 transfer of tolerance by freeze/thaw (F/T) extracts of tolerised sIg- leukocytes is described and some biological and

physico-chemical characteristics of the suppressive freeze/thaw extracts studied.

In chapter 5 the results obtained in this study are summarised and discussed in the context of other work in this field.

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CHAPTER I

Introduction

I. Clinical in vivo applications of monoclonal antibodies

Ehrlich (1) proposed in 1908 that antibodies specific for tumor antigens ("magic bullets") could be used to specifically eliminate malignant cells. The production of monoclonal antibodies (MAbs) by Kohler and Milstein (2) and subsequent developments (3) has opened the way for a wide range of clinical applications of MAb therapy: for example in kidney allograft rejection management (4-6), radioimmunoimaging (7) in cancer therapy (8-13), and in treatment of drug overdoses (14).

Allograft management

The Ortho multicenter transplant group (4) utilised the murine MAb OKT3 (which interacts with human T cells and inhibits their function) in treating the T cell mediated rejection of renal allografts. Patients undergoing acute renal rejection were treated with OKT3 daily for 14 days with a concomitant reduction in the doses of other immunosuppressive drugs. A similar group of patients received high-dose steroids. OKT3 reversed 94% of the rejections compared with 75% reversal for the group receiving steroids. One year graft survival was greater in the OKT3 treated group (62%) compared with that for the steroid treated group (45%). Antibodies (mainly of the IgG class) to OKT3 developed in 80% of patients either during or

after the second week of treatment with the drug. Host antibodies to OKT3 have also been reported by Tonkonogy et al (15) and by Dillman et al (16) There was no evident development of hypersensitivity, anaphylaxis or serum sickness in patients taking part in any of these studies.

Other MAbs have been used in therapy for allograft rejection (5,17,18) but with less striking results than those reported by the Ortho group (4).

Radioimmunoimaging

Larson et al (19) have used ^{125}I and ^{111}In conjugates of antimelanoma MAb to image metastatic melanoma. Mach et al (20) and Ferrands et al (21) have imaged colon cancer using ^{131}I-anti-carcinoembryonic antigen MAbs. Many animal models have also shown the potential for radiolabelled MAbs in imaging; for example Colcher et al (22) using ^{131}I-labelled anti-breast carcinoma MAb to image athmic nu/nu mice bearing human breast cancer cells. Ballou et al (23) using ^{131}I-MAb in Balb/c mice bearing teratocarcinoma, and Houston et al (24) using ^{125}I-labelled anti-Thy 1.1 MAb for lymphocytes in AKR/J mice. If multiple episodes of imaging are required in order to follow the progress/regression) of tumors, then the induction of anti-radiolabelled MAb antibodies may result, i.e., from exposures to xenogeneic MAbs.

Cancer therapy

(a) Monoclonal antibodies per se

Several studies have been reported on the use of MAbs in cancer therapy; Dillman et al (25,26) in the treatment of chronic lymphocytic leukemia and cutaneous T cell lymphoma, Miller et al (27-30) in the treatment of T cell leukemia, T cell lymphoma and B cell lymphoma, Sears et al (9,31) in the treatment of gastrointestinal tumors, Nadler et al (32) in the treatment of a B cell lymphoma, Ritz et al (33-35) in the treatment of acute lymphoblastic leukemia and lymphoma, Jaffers et al (15,36).

Many of the MAbs currently available are derived from murine hybridomas and the multiple infusions of these antibodies required for effective treatment of patients, would be expected to elicit anti-MAb antibodies. That this is indeed the case has been demonstrated by reports of anti-MAb antibody induction (4,9,15,16,26-28,31,36,37) in humans and in sub-human primates (38). Shawler et al (37) showed that five out of ten patients suffering from cutaneous T cell lymphoma (CTCL) developed an immune response (both IgG and IgM anti-MAb antibodies) to the MAb (T101) used in therapy after multiple infusions. (However, none of the six patients suffering from chronic lymphocytic leukemia (CLL) who were given the same MAb, developed any anti-MAb antibodies). The presence of anti-MAb antibodies was associated with a diminished clinical response together with a measurable decrease both in the number of target cells killed and in the level of circulating MAb. Multiple

infusions were associated with elevated titers. Some of the anti-MAb antibodies were shown to be specific for the variable region of the MAb. The titer of anti-variable region antibodies increased as the number of infusions of MAb increased. In two patients more than 50% of the response wwas anti-variable region.

The presence of low levels of anti-variable region antibodies has also been documented by Miller et al (27,28), by Sears et al (9) and by Jerne et al (38). Sarnesto et al (39) found that in mice immunised with a human IgG myeloma, 43% or the resulting anti-myeloma antibodies were specific for the variable region of the myeloma. However, this was not the case in mice receiving injections with two distinct myelomas. Similar findings have been reported by Ben-Nariah et al (40) using murine myelomas injected into rabbits. However, Tonkonogy et al (41) and Lowe et al (42), using animals immunised with polyclonal immunoglobulin preparations, were unable to detect anti-variable region antibodies. Thus, from the limited data available, it appears that multiple infusions of a single monoclonal antibody results in a substantial anti-variable region response; whereas immunisation with two or more (i.e., a polyclonal population) myelomas results in predominantly anti-constant region antibodies. Stratte et al (43) reported the presence of anti-variable region antibodies in subhuman primates immunised with murine MAb. Shawler et al (37) considered all available data on anti-murine MAb antibodies (as of August, 1985) and concluded that 41% of CTCL patients, 89% of gastrointestinal adenocarcinoma and 73% of renal

graft patients developed anti-MAb antibodies during therapy. 100% of healthy chimpanzees were found to produce anti-MAb antibodies when immunised with the MAb, Leu-1. The Leu-1 MAb is a murine derived antibody produced by Becton Dickinson.

(b) Monoclonal antibody conjugates

Patients suffering from cancer or undergoing renal rejection, who have been treated with MAb, are generally immunosuppressed as a consequence of the disease itself and/or because of immunosuppressive therapy; and consequently may not be as responsive to foreign antigens as healthy people. Therefore, in order to achieve a cytotoxic effect, various cytotoxic substances (e.g., radioisotopes and cytotoxic drugs) have been attached to MAbs.

MAb-ricin-A chain conjugates for use in humans have been made where MAb represents (a) anticolorectal carcinoma (44,45), (b) anti-p97 antimelanoma (46) and (c) anti-CALLA (47). When tested in vitro the ricin A-chain moiety of these conjugates was found to be almost as toxic as that of the whole ricin molecule (48). Youle and Neville (49) have used cytotoxic conjugates of MAb and the whole ricin molecule rather than just the A-chain. Krolick et al (50), using a mouse system, have shown that ricin-MAb conjugates are more cytotoxic than MAb alone.

Several conjugates of MAbs coupled to cytotoxic agents are currently being investigated, including conjugates of MAbs with methotrexate (51), adriamycin (52) and chlorambucil (53,54). Whilst

most of the work in this area has been in animal model systems, Ghose et al (55), have treated humans with chlorambucil non-covalently bound to goat or rabbit antimelanoma antisera and reported improvements in 2 out of 13 patients in the study

It is apparent that MAbs and MAb conjugates are of use in many areas of medicine. A major problem, the formation of anti-MAb antibodies in patients undergoing MAb or MAb-conjugate therapy, must be solved in order to prevent a decrease in the effective dose of MAb reaching the target cells and to prevent the deleterious consequences of antibody-MAb complex formation. A strategy for achieving this goal is to induce specific immunological tolerance to the MAb in patients before they begin MAb or MAb-conjugate therapy.

II. Immunological tolerance

Definitions

A genetically controlled inability to respond or an inherent lack of immunogenicity in a given molecule does not constitute active tolerance. Tolerance is the specific suppression of the immune response induced by previous exposure to antigen. This suppression of the immune response may be achieved in various ways as described below.

A state of unresponsiveness to self-antigens (learned during the ontogeny of the immune system and maintained as part of an ongoing process) is observed in normal persons. This process is also

sometimes referred to as natural or self-tolerance in contrast to experimentally induced or acquired tolerance. There are numerous substance in the body that the immune system may become unresponsive to. These include cell surface antigens (especially those under MHC control) and antigens existing free of cells (e.g., foreign prooteins, viruses et.c.).

Tolerance to non-self antigens can also be acquired or induced in both immature and mature animals. Maturing animals pass through a period of responsiveness to induction of tolerance during which they are readily tolerized when exposed to antigen. It is still possible to induce tolerance beyond this period but it is more difficult. The length of this period varies with species: in man it is over before parturition. However, in mice it lasts until a few hours after birth. The duration of this period is also dependent upon the type of antigen to which tolerance is induced. For example, the "weak" antigen H-Y has a tolerance responsiveness period extending for two weeks post-partum compared with the "strong" H-2 antigens for which the period ends immediately after birth. Tolerance to weak antigens can be induced with smaller doses of antigen than are required to induce tolerance to strong antigens.

Induction of tolerance to non-self antigens in mature animals generally requires very large doses of antigen and/or exposure over a long period of time. It may be facilitated by artificially impairing the host's immune system by X-ray irradiation, thoracic duct drainage, treatment with immunosuppressive drugs or anti-lymphocytic

serum et.c.. Tolerance can then be induced by injection of antigen (this may be in a physical form and by a route, that favours tolerance as opposed to immunity, e.g., deaggregated gamma globulins); transfer of spleen, bone marrow or lymph node cells; by parabiotic fusion; and by production of radiation chymerae (by injection of thymectomised and lethally irradiated animals of strain A with T cell depleted bone marrow cells derived from strain B).

Whether or not tolerance has been induced can then be assessed by challenging the animal with the normally immunogenic dose/form of the antigen. This could be by injection of antigen with or without adjuvant and measurement of any antibody response; by placing a skin graft on the host; by inability of host cells to mount mixed lymphocyte reactions (MLR) in vitro; or by cell mediated cytotoxicity (CML). However, inconsistancies may arise between these different "measures of tolerance", e.g., animals may show tolerance to skin grafts and negative MLR and CML but others may give positive MLR and CML but show no rejection of skin grafts. Induced tolerance may not be absolute but only partial, such that, in time, a return to immunity and elimination of the antigen is observed. The relationship between naturally and experimentally induced tolerance is uncertain (56).

The demonstration of acquired tolerance requires at least two exposures to antigen: an initial tolerance inducing exposure i.e., with the antigen in a suitable dose and/or physico-chemical state and a subsequent challenge with the antigen in a suitably antigenic

state and/or dose. Tolerance can be total viz, there are no detectable antibodies to the antigen or partial viz, a reduction in the number of antibodies. Tolerance may be observed as a reduction in some or all of the immunoglobulin isotypes and/or as a depression of delayed hypersensitivity. For example administration of a large dose of antigen i.v. concurrent with the injection of antigen in an emulsion with Freund's Complete Adjuvant (FCA), leads to the specific depression of delayed hypersensitivity and of IgG2 antibody production with little effect on the IgG1 antibody response. This has been referred to as immune deviation.

Tolerance may be observed to one epitope on a molecule but not to others on the same molecule. This has been called split tolerance. [see Antigenic Competition below]

Immunological paralysis is generally taken to refer to the specific suppression to pneumococcal polysaccharide or to other non-metabolisable antigens. Immunological paralysis (treadmill tolerance) was first observed by Felton (57) after injecting mice with 10 or 100 micrograms of pneumococcal polysaccharide type III and then challenging them with virulent pneumococcus bacteria. Only mice receiving the lower dose were protected against disease. Mice receiving 100 micrograms were not protected due to the high dose of antigen paralysing the immune system. In reality these latter groups of mice do produce antibodies, but these combine immediately with the polysaccharide and are effectivley neutralized. Immunological paralysis could be considered as a kind of pseudo-tolerance.

Historical perspective

In 1911 Wells and Osborne (58) reported that feeding animals a large dose of antigen resulted in a decrease in their capacity to become anaphylactically sensitive to the antigen. Glenny and Hopkins (59) reported a decrease in the immune elimination of heterologous gamma globulin by rabbits previously given a large dose of antigen. Sulzberger (60) and Chase (61) showed the importance of route of administration in determining whether or not nominally antigenic materials would produce a state of unresponsiveness.

Owen (62), reported the acquisition of unresponsiveness to allogeneic antigens in dizygotic cattle twins. Fraternal cattle twins often share a vascular supply during intrauterine development, facilitating the exchange of blood elements and the establishment of a chimeric state. Thus acceptance of skin allografts between these dizygotic twins was observed.

In an attempt to account for these various observations Burnet (63) postulated his theory of clonal selection, which proposes that an unimmunized individual possesses a large number of lymphocyte clones. Each of these clones is derived from a single parent cell. Lymphocytes of a given clone can produce antibodies of only one specificity (clones may cross-react with a given antigen but with different determinants and/or with different affinities). Lymphocytes carry antigen specificity information on their surfaces (for a B cell this is an immunoglobulin and for a T cell it is encompassed within the T cell receptor). When an antigen enters the

body it is bound by a clone of cells bearing molecules specific for this antigen. This binding results in stimulation of the clone to expand and in the case of B cells to secrete antibodies specific for this antigen. A given antigen therefore selects a clone from the great number present in the body. Clonal diversification occurs during the late stages of fetal development through somatic mutation of immunoglobulin genes. Some of these mutations result in clones that are specific for self molecules. These clones are arrested in their development and eliminated (modern modifications of this theory view these clones as being functionally deleted, i.e., the clones may not be physically eliminated but are still present in the tolerant animal). If a non-self antigen is introduced into the fetus during the period of clonal diversification the immune system will eliminate the clone specific for that antigen. In effect, this antigen will now be considered as self by the immune system and the individual will be tolerant to that antigen. This theory was confirmed by the work of Billingham et al (64) on acquired tolerance to allogeneic skin grafts, and by Hasek et al (65) who found that embryonic parabiosis of chick eggs lead to a specific state of unresponsiveness. Further work by Hanan (66), Dixon (67), Simonsen (68) and Smith (69) using heterologous proteins and erythrocytes showed that in general the larger the dose of foreign antigen and the more immature the recipient, the more profound and longer lasting the tolerance. However, in most cases tolerance is not permanent i.e. animals eventually `recover' their immune function to the tolerising

antigen. It has become apparent that there exists a number of pathways towards unresponsiveness to self-components or the exogenous materials introduced experimentally (70).

General characteristics of tolerance induction

Dose of antigen (71-75), physical properties of the antigen (76-80), physiological state of the host (81-84) and genetic factors (85-87) may all influence the degree, duration and cellular basis of the tolerant state (56). Induction of tolerance will, in general, be favoured by any system that tends to decrease the magnitude of the immune response (88).

Mechanisms of tolerance induction can be divided into two main categories: central tolerance i.e. mechanisms that directly effect the functions of the immune system (clonal abortion and deletion, suppressor T cell mediated tolerance, antibody mediated tolerance, et.c.); and peripheral tolerance i.e. those mechanisms that do not directly effect the function of any of the cells of the immune system (treadmill tolerance). (A possible third category is immunological ignorance i.e. the antigen is not normally exposed to appropriate lymphatic drainage and stimulation of the immune response. However, this is not generally considered to be true tolerance, because the host never truly encountered the antigen).

Clonal deletion and abortion

Clonal deletion and abortion is the elimination or permanent

inactivation of specific T or B cells. Clonal deletion is formally considered to be responsible for tolerance if the tolerant state persists on transfer of cells from the tolerant animal into a recipient animal (or tissue culture) and no suppressor cell activity can be demonstrated. In addition culture of cells from tolerant animals in the presence of polyclonal B cell activators should not result in stimulation of specific antibody (89). The absence of clones cannot however be proven absolutely: this category may be due to the presence of functionally inactive clones rather than an absence of clones. The inactive, tolerized cells appear to mature in a normal fashion, but do not react to positive signals (interleukins et.c.) and/or they receive negative signals (suppressor factors). This state has been termed clonal anergy in contrast to clonal deletion.

Receptor blockade

Tolerance due to blockade is caused by interference by a tolerogenic form of antigen (or antigen-antibody complexes or anti-idiotype antibody) with the normal immune response; this could occur, in the simplest scheme, by blocking receptors for antigen and thus preventing antigen interacting with specific cells leading to an immune response. The unresponsive state is spontaneously reversible or reversible after transfer of cells into an antigen-free environment or following digestion of the antigen from the cell surface receptors.

Hellstrom and Hellstrom (90) reported that lymphocytes derived from mice bearing virally induced sarcomas were capable of killing these tumor cells in vitro. However, the addition of serum from tumor bearing mice to the in vitro culture blocked the killing of the sarcoma cells in a specific manner. Later studies showed that blocking was mediated by antibody-antigen complexes. A more general role for blocking factors in the maintenance of tolerance is unlikely because of the following observations: (a) sera from tolerant mice without demonstrable blocking activity in vitro, enhance tumor growth in vivo; (b) there is no correlation between blocking activity in vitro and tumor growth in vivo
(non-blocking sera are as effective in enhancing tumor growth as are blocking sera) and (c) the induction of tolerance is not facilitated by passive administration of blocking sera. Blocking factors (immune complexes) probably play only an auxiliary role in the induction and maintenance of tolerance.

T suppressor cells

Suppressor T cells induce tolerance by actively suppressing a cellular or humoral immune response. T cell regulatory networks or circuits have been described which involve antigen specific and idiotype specific T cells (91). Green et al (92) and De Kruyff et al (93) have demonstrated that the existence of contra-suppressor T cells which render T helper cells resistant to the suppressive effects of T suppressor cells; thus tolerance could result from a

decrease in contra-suppressor activity i.e. the regularly suppressed state of the immune system would be unaffected even after injection of antigen as T helper cells would not be activated.

Suppressor cells exhibit different specificities (antigen, allotype, idiotype, et.c.). Herzenberg et al (94) developed a mouse model demonstrating the mechanism of allotype suppression. BALB/c female mice that were homozygous for the Igh-1^a gene were crossed with SJL males which carry the Igh-1^b gene. The F_1 hybrids produced IgG2a molecules bearing both the Igh-1^a and Igh-1^b allotypes. However, if the mothers were immunized against the paternal allotype the expression of this allotype was chronically suppressed in more than half of the hybrids by the time they were six months old. This suppression was mediated by maternal antibodies to the Igh-1^b allotype which were able to cross the placenta and enter the fetal circulation. The suppression persisted long after the maternal antibodies had disappeared from the hybrid's blood. The transfer of spleen cells from normal into suppressed hybrid mice had no effect upon the appearance of Igh-1^b allotype. This indicated that suppression was an active process. The transfer of T cells together with spleen cells derived from suppressed hybrids into irradiated hybrids resulted in suppression of the Igh-1^b from the normal cells. Thus production of IgG2a by B cells required the action of allotype specific T helper cells. This interaction was modulated by suppressor T cells which were activated by the presence of maternal anti-allotypic antibodies in the fetal blood.

Anti-idiotypic antibodies can also induce activation of T suppressor cells. Eichmann (95) used A/J mice immunized with group A streptococcal carbohydrate to produce antibodies that bear a dominant idiotype (A5A) which were then purified from the mouse serum by affinity chromotography and then injected into guinea pigs. The IgG1 fraction of guinea pig anti-A5A was then separated from the IgG2 fraction. Guinea pig IgG2 anti-A5A antibodies were then injected into naive A/J mice which were challenged two months later with group A strepto -coccal carbohydrate. These mice showed suppression of anti-group A streptococcal carbohydrate antibodies that bore the A5A idiotype. Thus, the xenogeneic (guinea pig) anti-A5A serum reacted with the corresponding idiotype on the receptor of suppressor T cells which resulted in the latter's activation. The T suppressor cells acted upon T helper cells to actively suppress the anti-A5A response in an idiotype specific manner.

Antigen specific suppressor cells may also be induced by certain antigens. Kapp et al (96) used a synthetic polymer of l-glutamic acid, l-alanine and l-tyrosine (GAT) to demonstrate this type of suppressor cell. In mice the antibody response to GAT is genetically controlled. Strain C57BL/6 are high responders whereas DBA/A mice are non-responders unless the GAT is injected coupled to methylated bovine serum albumin. However, if mice are injected firstly with GAT alone and then with GAT coupled to methylated bovine serum albumin, no anti-GAT response is detected. Exposure to GAT alone elicits antigen specific suppressor cells which prevent T

helper cells from assisting the response of B cells to GAT coupled to methylated bovine serum albumin. The T cells recognise the methylated bovine serum albumin (carrier) and the GAT is recognised by B cells (hapten).

In the mouse mature T suppressor cells are Lyt-1^-, Lyt-2^+ whereas T helper cells are Lyt-1^+, Lyt-2^-. The result of T suppressor cell action is the functional depletion of T helper cells probably mediated via soluble lymphokines or suppressor factors which may consist of a Vh chain (antigen specificty) and a J chain. T helper cells control B cells and are themselves controlled by T suppressor cells. In addition T suppressor cell differentiation may be controlled by T helper/inducer cells.

Antibody mediated suppression

(a) High affinity antibodies

Dresser suggested that the production of small amounts of high affinity antibody following tolerance induction by antigen may also lead to tolerance induction (97). Passively administered antibody (especially high affinity) is known to be capable of specifically depressing the immune response (98). Probably the major mechanism of antibody mediated suppression is simply to rapidly clear the antigen, thus reducing or preventing the stimulation of an immune response. In addition production of antibody to antigen (or to passively administered antibody) may lead to the production of anti-idiotypic antibodies which may also have suppressive effects.

Antigenic competition

Certain antigens dominate over other antigens which are injected into animals simultaneously (dominance or suppresssion is a function of the relative proportion of each antigen) or sequentially (the antigen given first dominates; and in an optimal fashion if the injection of the second antigen corresponds with the peak antibody response elicited by the first antigen). The response to one antigen inhibits the response to the suppressed antigen (99). The effect is usually only observed for the primary response. Induction of tolerance to the dominant antigen abrogates the inhibition of response of the suppressed antigen. The underlying mechanisms for sequential competition is thought to be due to stimulation of a T suppressor factor by the first antigen and non-specific inhibition of the response to the second antigen by this factor.

There are two types of antigenic competition: intermolecular and intramolecular. In the former two competing antigens are present on two different molecules (e.g., globulin versus albumin, ferritin versus albumin, diptheria toxoid versus tetanus toxoid, et.c.). In the latter two competing antigenic determinants are present on the same molecule (e.g., the response of mice to rabbit IgG, i.e., when intact immunoglobulin is injected antibodies to the Fc portion predominate over antibodies to the Fab portion (a good anti-Fab response is obtained if mice are immunised with Fab alone)) (100). Competition is thought to occur at the B cell level, i.e., the clone that recognises the dominant determinant proliferates rapidly and

sequesters much of the antigen thereby depriving clones specific for the suppressed determinant of antigen.

Idiotype/anti-idiotype interactions

Jerne (103) has proposed that the immune system is a network of idiotypes and anti-idiotypes which normally exist in a state of dynamic equilibrium. The network hypothesis assumes that each lymphocyte is a part of a complex system in which every cell acts upon at least one other cell. This interplay between cells is via paratopes (which are the interaction sites of cells' receptors) and idiotypes. Consider, for example, a cell which bears a paratope (P1) which recognises an idiotype (I2) which is itself part of a paratope (P2) on another cell. The first cell may be activated by the interaction between its paratope (P1) and the idiotype (I2) on the second cell. However, the paratope (P1) itself bears an idiotype (I1) which is in turn recognised by a third cell that carries a paratope (P3) specific for idiotype (I1). This interaction may inhibit the first cell. These inter-relationships are assumed to hold for all lymphocytes. Each cell has the potential to be activated by recognition of an idiotype on another cell but this is counteracted by the inhibitory action of another cell recognising the first cell's idiotype. This results in a dynamic network of interacting cells.

When antigen is introduced into the system, this equilibrium is disturbed and specific idiotype bearing clones are stimulated to expand. The antigen carries an epitope, i.e., an antigenic

determinant, that resembles one of the idiotypes present in the network which then perturbs the network leading to immunity or suppression. Prior to the entry of the antigen there are as many paratopes as there are idiotypes and for every epitope there is a corresponding idiotype. Thus, every epitope has its own internal image in the body. The antigen bearing epitope (E1) is recognised by paratope (P1) which ordinarily recognises the idiotype (I1). ((I1) is the internal image of (E1)). The recognition of (E1) by the cell bearing (I1) provides an activating signal to the cell carrying this receptor. As (E1) is not an inherent part of the network there is no counteracting inhibitory action upon it. Therefore the balance between activating and inhibiting signals is temporarily disturbed in favour of activation and the cell begins to differentiate in the direction of an antibody secreting plasma cell. This disturbance "ripples" through the network but is eventually dissipated as it travels further from the originating event. Once the stimulation by E1 ceases the disturbed elements of the network return to equilibrium.

Thus, idiotype specific T and B cell clones are in turn stimulated to proliferate, and the system re-equillibrates, with a new distribution of clones; anti-idiotypic antibody can depress the production of antibodies bearing that idiotype and therefore maintains a state of specific immunological tolerance (102,103). (The network is postulated to work at the B cell, T cell and serum antibody levels). It has been shown that idiotype specific

suppressor and helper T cells can be produced (104,105) in some states of immunological tolerance.

It is possible that the network is not so homogeneous as suggested by Jerne but is instead more akin to a series of interconnected cell circuits

whereby each cell regulates the one preceding it and is itself regulated by the cell following it. For example, a T helper cell (Th1) recognises the idiotype of a T suppressor cell (Ts2) and also of a B cell. Ts2 recognises the idiotype of a second T helper cell (Th2). Th2 recognises the idiotype of Ts1; and Ts1 recognises the idiotype of Th1. If Th1 is activated first it facilitates the maturation of Ts2 which depletes Th2 in the circuit resulting in the inability of Ts1 to mature. This in turn results in an increase in Th1, such that the circuit is locked into help. However, should Th2 be activated first, it will facilitate the differentiation of Ts1 which depletes Th1, in turn this prevents maturation of Ts2 which means there is now no restriction to the differentiation of Ts2. In this instance the circuit is locked into suppression.

General scheme of antigen/immune system interactions

In order to evoke an immune response antigens must be presented to the immune system by antigen presenting cells (APC) i.e. macrophages, dendritic cells, Langherans cells and B cells. These cells present antigen in the context of MHC Ia region coded products to T helper cells which in turn produce antigen specific product(s) and various lymphokines (IL-2, IL-3, T cell replacing factor (TRF) and B cell

growth factor (BCGF) (105-108). Antigen/APC and/or T helper cell derived antigen specific factor(s) stimulates B cells to enter G_1 phase from G_0 and then to enter S phase following lymphokine and/or monokine mediated stimuli. These cells differentiate into plasma cells after stimulation from TRF and related substances (109-111). Tolerance, therefore, may be the result of lesions in any of these interactions i.e. APC/T cell, APC/B cell, T cell/B cell, and various lymphokine/monokine/cell interactions.

The role of IL-2 in tolerance induction

Conlon et al (112), Lowry et al (113), and Matzinger et al (114) have shown MHC restrictions operating in tolerance induction. Malkovsky and Medawar (115), observing that if the same molecule is recognised in the context of MHC as both an antigen and as a tolerogen then there must be another regulatory mechanism at work. They propose that the outcome of an interaction between antigen/MHC and T cells, depends upon the presence or absence of IL-2: if there is a deficit of IL-2 then tolerance is induced. In support of this hypothesis they cite the work of Cleveland and Claman (116) who found that injection of Con A (IL-2 stimulator) into mice tolerised with haptenated spleen cells changed the tolerogenic nature of haptenised cells into an immunogenic one. Malkovsky et al (117) showed that the administration of exogenous IL-2 abrogated the tolerogenic potential of semiallogeneic cells in neonates (which are lacking in IL-2 production.

III. Immunological tolerance with reference to gamma globulins

As stated above (see Objectives of study) this thesis examines the tolerogenic properties of conjugates of XIgG and monomethoxypolyethylene glycol. These conjugates were synthesised in an attempt at solving the problem of preventing anti-XIg antibody production in certain therapeutic applications of XIgG as opposed to the use of gamma globulins in general as model proteins in the investigation of immunological tolerance. Thus the remainder of this introduction will focus upon the induction of tolerance by gamma globulins.

The special nature of immunoglobulins as antigens

Immunoglobulin antigens differ fundamentally from other antigens in that they are antibodies and as such possess idiotypic, allotypic and isotypic determinants in addition to complement binding domains and the potential to interact with cellular Fc receptors. The observation that deaggregation of IgG renders it tolerogenic whilst aggregated IgG is immunogenic (76) lends support to the special status of immunoglobulins as antigens. Xenogeneic immunoglobulins may interact with elements of the host's immune system functioning in an active role as an antibody rather than simply being acted upon as a passive antigen. IgG1 and IgG3 activate the complement system once bound to antigen. IgG2 is less effective in this role. IgG1 and IgG3 bind to mononuclear, neutrophil, T and B cells and to platlets. IgG2 binds to T and B lymphocytes. IgG4 binds to neutrophils, T and

B lymphocytes. Xenogeneic immunglobulin may be able to cross react with host Fc receptors: thus it is possible that immune complexes composed of host and xenogeneic immunoglobulin facilitate unusual cellular interactions and juxtapositions whereby both immunoglobulins are interacting through their Fc moieties with the same or different clones or cell types. In this sense each immunoglobulin considers the other to be its antigen. Immune complexes bind to B cells via Fc and antigen receptor cross-linking resulting in inhibition of that clone. However, these immune complexes could also augment the immune response if, for example, the ratio of "antigen" to antibody is high by encouraging fixation of the antigen on certain antigen presenting cells. Antigens complexed with antibody show greatly enhanced localization in germinal centers. In general IgM antibodies tend to enhance the immune response whilst IgG tends to suppress if the above mechanisms are operative.

The xenogeneic antibody's variable and hypervariable regions may induce anti-idiotypic antibodies that closely resemble autochthonous idiotype/anti-idiotype network constituents. This may diminish any perturbation of the network resulting in a lack of an immune response.

Induction, kinetics and duration of tolerance

Dresser (79) demonstrated that changing the physical state of gamma globulins rendered them either immunogenic or tolerogenic; removing aggregated material by means of ultracentrifugation yielded

a tolerance inducing material, whereas heat-aggregation of HGG yields immunogenic material. the quantitative relationship between the aggregated and non-aggregated globulin injected is critical in determining whether immunity or tolerance is induced (118). Gamma globulins are T-cell dependent antigens requiring helper T cell function (119) and therefore when considering immunological tolerance, as measured by a lack of production of antibodies, lesions in either T or B cell populations must be considered as possible mechanisms.

Dresser (80) showed that doses of deaggregated BGG in the range 50 to 200 micrograms could induce tolerance when given i.p. into CBA mice. Golub and Weigle (120) showed that tolerance induction by HGG was dose dependent and dependent upon the experimental strain of mice injected. For example in the case of the parental strains of the BDF1 mice used in the work described here; as little as 50 micrograms of HGG could induce tolerance in C57BL/6 mice but at least 500 micrograms were required for DBA/2J mice. HGG was administered i.p. in both cases. A dose of 100 micrograms will induce unresponsiveness in thymocytes whilst 2.5 mg is required to induce unresponsiveness in bone marrow cells (121). This compares with 0.5 mg of DHGG required to induce tolerance in splenic B cells (122).

The kinetics of tolerance induction and persistence differ for each cell type: thymocytes become unresponsive within 1 day of administration of DHGG and unresponsiveness is found in splenic T cells within 1 to 2 days (123). This compares with an interval of 3

days for splenic B cells (124) and an interval of 8 days for bone marrow cells (125). Unresponsiveness (total) persists in the thymocyte and splenic T cell populations for a minimum of 120 days following tolerisation (126,127), compared with a period of unresponsiveness of 50 days for bone marrow cells and 45-50 days for splenic B cells (126). Thus, there is a period of about 70 days (120-50 = 70) when the T cell population is tolerised but the B cell population is not.

Mechanistic considerations

(a) Receptor blockade

If receptor blockade is responsible for tolerance to HGG then antigen reactive B cells would be present in tolerised animals. However, the injection of LPS (a polyclonal B cell stimulator) into tolerised animals does not result in production of plaque-forming cells to HGG (127). However, many of the B cells stimulated by polyclonal activators are cells producing low affinity antibody. Thus the majority of polyclonally stimulated B cells may actually be untolerised but capable of binding antigen to some extent (128).

(b) Suppressor cells

HGG specific suppressor cells have been reported by Basten et al (129) and Benjamin (130). The suppressor cell has been characterised as a T lymphocyte (131) that is Theta, Ia and Ly-2 positive (132,133). The HGG specific T cells suppress both B and T cells

specific for HGG (131).

(c) Soluble suppressor factors

Soluble suppressor factors have been described in association with HGG specific suppressor cells. Jones and Kaplan (134) showed that suppression could be mediated by a soluble factor obtained from the spleen cells of tolerised donors. This factor was at least partly protein and had a molecular weight in the order of 45 kD. The factor was HGG specific but not anti-HGG specific. Taniguchi and Miller (135) confirmed the specificity observations and reported a molecular weight range for their factor of 30-55 kD. They also showed that it did not bear any classical immunoglobulin determinants and was a product of the I region of the MHC (it carried Ia determinants). Similar observations were reported by Chaout (136). In the above models tolerance was induced with 2.5 mg of DHGG.

Relevance of T suppressor cells and factors

The presence of T suppressor cells in tolerance to HGG has been described above. However, their relevance to the maintenance of tolerance to HGG has been questioned. One of the major incongruities is the observation that the kinetics and magnitude of T suppressor cells do not parallel the time course of tolerance. (129,137,138); viz T suppressor cell activity is lost 40 days after tolerisation but splenic T cells remain tolerised for 120 days and splenic B cells for up to 80 days.

Parks et al (139) were able to tolerise nude (athymic) mice with 2.5 mg of DHGG. They also observed that the duration of tolerance was greater in athymic mice (131 days) than in normal animals (70-80 days). If T suppressor cells are indeed responsible for maintenance of tolerance then it would be expected that the duration of tolerance in athymic mice would be shorter than that in normal mice rather than the other way around.

Benjamin (140) found no evidence for HGG specific suppressor cells in neonates tolerised through colostral administration of tolerogen but did find HGG specific B cell unresponsiveness. Benjamin (141) also reported a lack of suppressor cells in mice tolerised with low dose of tolerogen (100 micrograms), although the mice were made unresponsive. He also showed that HGG specific suppressor cells cannot be reinduced in tolerised animals after the disappearance of the initial transient suppressor cells i.e. suppressor cells could not be generated by secondary tolerisation.

Parks et al (127) reported that colchicine could inhibit T suppressor cell generation in mice tolerised with DHGG without effecting the state of tolerance induced by the DHGG in either the T or B cell populations.

Thus some workers (127) regard the presence of T suppressor cells as regulatory phenomena of no relevance to the maintenance of tolerance. Loblay (143) believes that the apparent incongruity between the kinetics of T suppressor cells and the duration of tolerance to HGG can be resolved by his demonstration of the

existence of T suppressor cells existing in two independent states: as effector cells and as memory cells. Following tolerance induction effector T suppressor cells are transiently produced and are the mediators of the primary suppression. T suppressor memory cells persist after the T suppressor-effector cells have disappeared and are only detectable following secondary antigenic stimulation. Primary suppression was detectable 4-5 days following tolerisation and peaked 7-14 days later. Subsequent antigenic challenge resulted in the appearance of a classical suppressor response, indicating the presence of memory suppressor cells. These cells were shown to be antigen specific and phenotypically similar to the primary effector T suppressor cells i.e. Ly-1-2,3+, Thy-1+, Ia+. These memory cells could be stimulated for at least 9 months after primary immunisation. The secondary effect displayed all the characteristics of a classical anamnestic response i.e. tolerance showed accelerated onset, was more profound and longer lasting. In the absence of antigenic stimulation T suppressor memory cells remain functionally latent and therefore are not detected in the usual type of transfer experiment used to detect T suppressor cell activity. However, immunogenic challenge of the tolerant animal leads to reactivation of these cells and the development of suppressive activity. Spontaneous recovery from the tolerant state is assumed to be due to the death of T suppressor memory cells over time. Loblay explains the observations described above that were presented as evidence against a role for T suppressor cells by suggesting that the data should be reinterpreted

as showing a dissociation between the primary appearance of effector T suppressor cells and the induction of long lived memory cells rather than as a dissociation between tolerance and suppression; a single dose of 100 micrograms of DHGG was unable to generate detectable primary suppression but an anamnestic T suppressor response could be elicited by challenging with AHGG after tolerising with only 1-10 micrograms of DHGG.

Whether or not Ts memory cells are the sole mechanism of tolerance maintenance is not known. T helper and B cells could also be deleted or inactivated. However, thoracic duct lymph from, HGG tolerant mice has been shown to contain functional T helper memory cells (143). In addition, despite reports of direct inactivation of B cells by antigen (144,145) it is impossible to be certain that all T suppressor cells were removed; for example it is difficult to kill all T suppressor cells using anti-Thy-1 and complement (143). In addition the nude mice experiments discussed above may be invalid as it is now known that nude mice do possess some T cell functions including specific suppression (146).

IV. Polyethylene glycol conjugates

Enzyme-$(PEG)_n$ conjugates

Abuchowski demonstrated that bovine serum albumin (BSA) and catalase lost their immunogenicity when covalently attached to polyethylene glycol (147-149) using 2,4,6-trichloro-s-triazine

(cyanuric chloride) as the coupling agent. The amount of PEG attached to an enzyme can be varied by varying the molar ratio of activated PEG to amino groups in the reaction mixture and by increasing the pH at which the reaction is performed (150). Several enzyme$(PEG)_n$ conjugates have been described: PEG conjugated to uricase (150), to phenylalanine ammonia lyase (150), to trypsin (151), to glutaminase (152), to arginase (153) and to adenosine deaminase (150) in addition to the conjugates mentioned above. As increasing amounts of PEG are attached to an enzyme the latter's ability to bind anti-enzyme antibodies is reduced. The degree of substitution of PEG can be increased to such an extent that no antigenicity remains as measured by Ouchterlony double gel diffusion or in vivo. However phenylalanine ammonia lyase was an exception, remaining antigenic regardless of the degree of substitution (150). Enzyme$(PEG)_n$ conjugates are able to pass membrane barriers and equilibrate with extravascular fluids (150). The repeated administration of the conjugates did not lead to accumulation of PEG in organs or tissues, nor was there evidence of toxicity (148).

Tolerogenic conjugates of PEG

On the basis of the work of Abuchowski et al (148,149) showing that certain proteins lost their immunogenicity following conjugation with mPEG and that the clearance rates of these conjugates were significantly lower than those of the unconjugated protein Lee and Sehon (154) hypothesised that mPEG conjugates of various proteins

antigens would be tolerogenic. Lee and Sehon coupled ovalbumin (OA) and the non-dialysable constituents of RAG to PEG and showed these conjugates to be nonantigenic, nonallergenic, nonimmunogenic (154-157) and capable of specifically suppressing the primary or secondary IgE response to the unmodified antigen. Treatment of rats with $OA(mPEG)_n$ conjugates prevented these animals from mounting an anti-OA IgE response and protected them from anaphylactic shock (154). Clinical trials of $HBV(mPEG)_n$ or $RAG(mPEG)_n$ in the treatment of patients allergic to HBV or RAG demonstrated that IgE levels were reduced and general symptoms showed amelioration (158). Similar effects were observed in patients after 5 months of $HBV(mPEG)_n$ immunotherapy (159).

The mechanism of tolerance induction by these conjugates was investigated. It was shown that spleen cells from tolerised mice could transfer this tolerance when injected into X-irradiated (550 rads) mice (156); T suppressor cells were shown to be involved in the transfer of suppression (160). Treatment of the recipient mice with cyclophosphamide 2 days prior to cell transfer abrogated the suppressive effect; thereby, dispelling the possibility that transferable suppression was simply due to the carry-over of tolerogen rather than due to the T suppressor cells (161). Incubation of spleen cells, taken from mice tolerised with $OA(mPEG)_5$ (average molecular weight of mPEG = 10,000), with Sepharose-OA conjugates at 37^0C for 5 hr, resulted in the release of a soluble factor(s) which, when injected into naive syngeneic recipients, was 1

able to induce suppression to the unmodified antigen (162). Peeters and Carter (163) demonstrated that persisting IgE antibodies were probably due to a long lived B cell that was refractory to radiation in the range 600-1,000 rads). Thus, two populations of B cells may be envisioned: one of which is refractory to tolerising protocols and another which is tolerisable. In this model tolerance is considered to be due both to the indirect action of T suppressor cells (either per se or via soluble factors) and to the direct inactivation of high-affinity B cells.

The general applicability of this method for conversion of proteins to tolerogens was clearly demonstrated by Wie et al (164) by synthesising a series of conjugates of OA, Timothy grass pollen, RAG, Dog albumin and bovine pancreatic ribonuclease using mPEG of average molecular weight 2,000, 5,000, 10,000 and 20,000. All these conjugates were able to reduce the IgE antibody response in sensitised animals when further injections of the sensitising antigen were given. In addition all these conjugates had either no or greatly reduced antigenicity (155,157). Similar findings were reported by King et al for their conjugates of antigen E (of RAG) and mPEG (164,166). The degree of substitution (i.e., the average number of mPEG per protein molecule) was in general inversely related to the average molecular weight of the mPEG for a given series of conjugates of the same protein; thus a highly tolerogenic conjugate required a smaller number of high molecular weight mPEG than would be required if the mPEG were of lower molecular weight.

IgG(mPEG)$_n$ conjugates

Suzuki et al (167) coupled polyethylene glycol to human IgG obtained from pooled plasma and to anti-hepatitis B virus surface antigen. From immunoelectrophoretic and circular dichroism studies of their conjugates it was concluded that no alteration of secondary or tertiary structure of the IgG molecule had occurred as a consequence of covalently attaching PEG. A small shift toward the anode was observed when an IgG(PEG)$_{25}$ sample was immunoelectrophoresed; this was probably due to the loss of e-amino groups (cationic) of lysine residues which are consumed in the coupling procedure. Evidence that the Stokes' radius increased as a result of the attachment of PEG was provided from PAGE (the migration of the conjugate was markedly reduced as the degree of substitution of the conjugates increased) and from high-performance size exclusion chromatographic analysis (the conjugate eluted earlier than unconjugated IgG as the degree of substitution increased. The state of aggregation of the conjugates was studied (Dresser (76) showed the importance of the degree of aggregation in determining the outcome (tolerance or immunity) of administration of IgG.) by examining the interfacial aggregation of IgG. IgG is susceptible to aggregation at an air/water or solid/water interface (168). Samples were shaken in glass tubes for 90 mins to induce aggregation. Native IgG showed about 50% aggregation compared with about 12 % for IgG(PEG)$_5$ and 2-3% for IgG(PEG)$_{25}$. Aggregation by heating was also studied; incubation of native IgG at 60 ^{0}C for 2 hr resulted in 18% aggregation whereas the

$IgG(PEG)_{25}$ conjugate showed 9% aggregation. However, two other conjugates, $IgG(PEG)_2$ and $IgG(PEG)_5$ showed the same degree of aggregation as IgG per se. The molecular weight distribution of these heat-aggregates was investigated by passage through a G4000SW-type high performance size-exclusion chromatography column. The heat treatment of IgG per se resulted in the formation of aggregates over a wide range, with the peak weight at $> 7 \times 10^6$ D. However, with aggregates of the conjugates the peak molecular weight decreased as the degree of substitution increased. Thus, the attachment of PEG to IgG reduces the amount of aggregation.

The effect of conjugation upon the ability of the IgG to bind antigen was also studied using IgG anti-HBs and HBs; the HBs binding ability of the conjugates decreased as the degree of substitution increased, i.e., the conjugation of the antibody decreased its antigen binding capacity.

CHAPTER II

Synthesis and tolerogenic properties of conjugates

Introduction

General materials and methods will be described followed by details of the synthesis, purification and chemical characterisation of IgG(mPEG)$_n$ conjugates and sham-conjugated controls. Experiments concerning the tolerogenic poperties of conjugates as a function of dose and of the degree of substitution with mPEG will then be described. Further experiments utilising the optimally substituted conjugate (with respect to tolerogenicity) will be presented. These include investigations of the in vivo fate of the conjugate and the kinetics of tolerance induction by the conjugate. The effect of multiple injections of conjugate upon tolerance induction will be discussed. Finally, experiments examining the spectrum of tolerance specificity will be presented.

Materials and Methods (General)

Certain procedures and techniques were used throughout the present work and were performed as described here unless otherwise stated in the text.

Animals.

Female BDF1 mice 6-8 weeks old were used unless otherwise stated. All mice groups consisted of 3 individuals unless otherwise stated. Tables longer than one page show data from concurrent experiments.

Antigens and immunisations.

Two human myelomas IgG#1 and IgG#2 were obtained courtesy of Dr. G. Delespesse and Dr. F. Paraskevas. Each of these was precipitated twice from serum with a 50% solution of ammonium sulphate, spun at 3000 rpm for 30 minutes at 40 C and dialysed against 0.05M Tris-HCl pH = 8.0. It was then passed through a DEAE cellulose column equilibrated in the same buffer. Fractions from the peak obtained were pooled and divided into two aliquots one of which was dialysed against borate buffer pH = 9.6. and the other dialysed against PBS pH = 7.4. Some of the latter aliquot was heat-aggregated using the method of Chiller and Weigle (124). A solution of 20 mg/ml DEAE purified HGG was heated at 63^0C for 25 min with occasional stirring. The HGG was incubated in an ice bath for 12 hours and then precipitated by the dropwise addition of 2.18 M Na_2SO_4 until a final concentration of 0.62 M Na_2SO_4 was reached. The precipitate was again incubated on ice for 30 min and then washed three times with 0.62 M Na_2SO_4 before being dissolved in PBS. The concentration of the aggregated HIgG was determined by the biuret method (169). The material was aliquotted and stored at -20^0C. Mice received a 10 micrograms injection of haIgG#1 or 50 micrograms of haIgG#2, i.p. (contained in a 3 ml disposable syringe fitted with a 26 gauge needle) in 0.5 ml PBS.

Aggregated ovalbumin AOA was obtained by heating to 63^0 C then cooling on ice (this heating and cooling was carried out a total of three times) the monomeric fraction of five-times recrystallised OA

obtained by passage through an AcA-54 gel filtration column. Mice received 50 micrograms of AOA, i.p. in 0.5 ml PBS.

Induction of tolerance to haIgG.

Mice were warmed with heat-lamps for 3 to 5 minutes and then injected i.v. into either the left or right tail vein using a 1 ml disposable syringe (fitted with a 27 gauge needle) containing 100 micrograms HIgG$(mPEG)_n$ conjugate in 0.5 mls PBS pH = 7.4. except where otherwise indicated. The interval between tolerisation and immunisation was 6 days unless otherwise stated.

Enzyme-Linked Immunosorbent Assay.

96-well microtitre plates (Costar) were coated with 100 microlitres (at 10 micrograms /well) of Human IgG derived from Human Gamma Globulin (Capell laboratories) that had been dialysed against 0.05 M Tris-HCl pH = 8 and purified by column chromatography on DEAE-cellulose equilibrated in the same buffer. Following dialysis against carbonate-bicarbonate buffer pH = 9.6., the IgG was aliquotted and stored at -20. Following overnight incubation at 4 ^0C, the plates were washed 3 times in PBS-$Tween_{20}$, pat-dried and 100 microlitres/well of blocking buffer added (1% BSA in PBS). Plates were stacked (the top-most being sealed with a plastic adhesive cover) and placed in a non-transparent, closed box containing wet paper towels, throughout all incubation periods. After 1 hour at RT the plates were washed and dried as before then test sera diluted in 0.5% BSA/0.5% $Tween_{20}$ in PBS, were added (100 microlitres/well). As

titer values were derived by interpolation sera were two-fold serially diluted, then incubated at RT for 2 hours. Plates were again washed and dried, then alkaline phosphatase conjugated goat anti-mouse antibody (Zymed Inc.; IgG1 specific in the case of experiments involving HIgG$(mPEG)_{20}$ and IgG specific in the case of experiments involving HIgG$(mPEG)_{17}$) was added at 1:1000 dilution, at 100 microlitres/well and incubated in the dark for 2 hours at RT. The substrate (p-nitrophenol phosphate, Sigma Chemical Co.) was added at a concentration of 1 mg/ml in diethanolamine buffer and incubated for 30 min. The enzyme-substrate reaction was stopped by the addition of 100 microlitres of 3 M NaOH to each well and the plates read at 405 nm and a reference wavelength of 630 nm on a Dynatech MR 600 microplate reader.

Computer acquisition and processing of ELISA data

The ELISA reader was connected to a Tandy 1000 (IBM compatible) personal computer via an RS-232-C serial interface and a null-modem device. Data were either stored or directly processed by removal of the header sequence (i.e. the text information placed at the begining of each plate for manual recording of information on ELISA print-out tape) Reformatting of the data in an 8 x 12 array corresponding to a 96-well plate was performed; and the values of the standard curve for the current group of plates compared with average values of the standard curves from previous runs. Values of OD were plotted against the log of the reciprocal of the dilution. Points on the linear

portion of the curves were selected and the best straight line obtained by the method of least squares. Titers at 0.3 OD were calculated from this line. A value of 0.3 OD units was chosen because it lay on the linear part of the titration curve and was twice the average background value. These data were then directly read into spreadsheets for processing and graphical representation. This system allowed for the rapid processing and analysis of large numbers of plates e.g. 25 plates in minutes compared with hours of graphing-out of data by hand.

Buffers

Borate

9.525 g of $Na_2B_4O_7.10H_2O$ (0.025 M) was dissolved in 1 litre of doubly distilled water . 50 ml of this solution was mixed with 11.1 ml of 0.1 M NaOH and the solution made up to 100 ml with doubly distilled water .

Carbonate-bicarbonate

0.795 g of Na_2CO_3 (0.0075 M) and 1.465 g of $NaHCO_3$ (0.0175) were dissolved in approximately 400 ml of doubly distilled water and the pH adjusted to 9.6. The solution was made up to a total volume of 500 ml with doubly distilled water .

Diethanolamine

19.4 ml of diethanolamine and 20.2 mg of $MgCl_2.6H_2O$ (0.1 M) were

dissolved in 140 ml of doubly distilled water and the pH adjusted to 9.8. The solution was made up to a total volume of 200 ml with doubly distilled water . The solution was stored at 4^0C and discarded after 3 weeks.

Phosphate buffer

2.62 g of K_2HPO_4 (0.015 M) and 13.41 g of KH_2PO_4 (0.01 M) were dissolved in approximately 300 ml of doubly distilled water and the pH adjusted to 7.5. The solution was made up to a total volume of 500 ml with doubly distilled water .

PBS

Twenty times concentrated PBS was prepared and diluted for use as required as follows: KH_2PO_4 (9.8g = 0.072 M), NaCl (320 g = 5.5 M), $Na_2HPO_4.H_2O$ (87.6 g = 0.55 M) were dissolved in doubly distilled water . The pH was adjusted to 7.4 and the solution made up to a total volume of 2 litres with doubly distilled water.

Tris-HCl

121.17 g of $C_4H_{11}NO_3$ (1 M), 6.057 g of Tham and 8.8 g of NaCl (0.15 M) were dissolved in approximately 500 ml of doubly distilled water . The pH was adjusted to 8.0 and the solution made up to a total volume of 2 litres with doubly distilled water .

Radioiodination of proteins by the Chloramine T method

The method described here is based on that of McConahey and Dixon (170). Amounts indicated below are for 50 micrograms of protein. All

buffers and solution were freshly prepared. The protein was placed in a 1.5 ml Eppendorf plastic vial and to this was added 0.5 mCi of ^{125}I, 0.25 microlitres of 0.5 M phosphate buffer (pH 7.5), 25 microlitres chloramine T at a concentration of 0.05 mg/ml in 0.05 M phosphate buffer (pH 7.5). The mixture was vortexed for 45 sec and then 100 microlitres of Na_2SO_4 added at a concentration of 2.4 mg/ml in 0.05 M phosphate buffer (pH 7.5). The tube was briefly vortexed and then 200 microlitres of KI added at a concentration of 10 mg/ml in 0.05 M.

The reaction mixture was then applied to a 10 ml disposable Sephadex G 25 column equilibrated in PBS. The radiolabelled protein was separated from the unbound iodine, pooled and its specific activity determined. Adjustment of activity was made to take into account the decay of the isotope using physical decay tables with time. The radiolabelled protein was aliquotted and stored at -20^0C. Radiolabelled protein was discarded after 3 weeks.

Characterisation of conjugates

The degree of substitution i.e., the average number of mPEG groups per HIgG molecule was determined by nuclear magnetic resonance (NMR) spectroscopy and biuret analysis.

(a) NMR analysis

An aliquot of conjugate (2-5 mg) was dialysed against distilled water in order to remove salts, lyophilised, then dissolved in D_2O (0.5 ml for the 90 MHz NMR machine and 1 ml for the 300 MHz NMR

machine). The proton ^{1}H-NMR spectra were then determined using either a Nicolet modified Bruker WH 90 DS or a Bruker AM 300 NMR spectrometer operating at 90 MHz and 300MHz respectively. Samples of mPEG of known weight served as standards. The peak for mPEG in each sample and the peaks for the standards were integrated and the amount of mPEG present in the samples determined. (The NMR machines were operated by Mr. K. Marat, Dept. of Chemistry, University of Manitoba).

(b) Biuret analysis

The s-triazine cross-linking group of the mPEG intermediate absorbs in the 280 nm range of the electromagnetic spectrum thus making direct determination of the protein content of the conjugates by OD measurement, difficult. Therefore protein content was determined by the biuret method of Dittebrandt (169). All protein determinations included a standard curve composed of dilutions (0.125-2.5 mg) of DEAE purified HIgG (Capell Laboratories). To 1 ml of saline, sample or standard, 2.5 ml of biuret reagent ($NaKC_4H_4O_6.4H_2O$ (9.0 g); $CuSO_4.5H_2O$ (3.0g) KI (5.0 g) and NaOH, 0.2 N, carbonate-free, to 1 litre) was added, the tubes vortexed and left for 30 mins at RT. Tubes were read at 555 nm against water commencing with two reagent blanks then the standards and samples and finally another two reagent blanks. The four blanks were included so that the length of time taken to measure the OD of the tubes could be controlled for. The average OD of the four reagent blanks was

subtracted from the average OD of the duplicate tubes of standards and samples and the resulting data were used to determine the protein content of the samples either by manual plotting of the data or by computerised linear regression.

Statistical Methods

Statistical analyses were performed using the analysis of variance, Dunnet's test, simultaneous confidence intervals, Bartlett's test for equality of slopes and tests for equality of intercepts. Experimental groups differing significantly ($P < 0.05$) from control groups are marked with a * in the tables.

Synthesis of conjugates and sham-conjugated control

Introduction

Abuchowski (149) used cyanuric chloride to couple PEG to various enzymes at a pH of 9.3 at 4^0C, overnight. Jackson et al (171) have described conditions for synthesis and characterisation of OA-$(mPEG)_n$ conjugates. Active mPEG intermediate, (2-O-mPEG-4,6-dichloro-s-triaz-ine) was synthesised by Dr C-J. C. Jackson. In the present work the conjugation conditions differed from those of Jackson et al in so much as the immunoglobulin conjugations were carried out at a pH of 9.6 (rather than 9.2) and the conjugation was performed entirely at 4^0C (rather than 4^0 C for half an hour followed by half an hour at RT). In addition the molarity of borate was 0.025 M in the present work.

Materials and Methods

Preparation of the mPEG intermediate

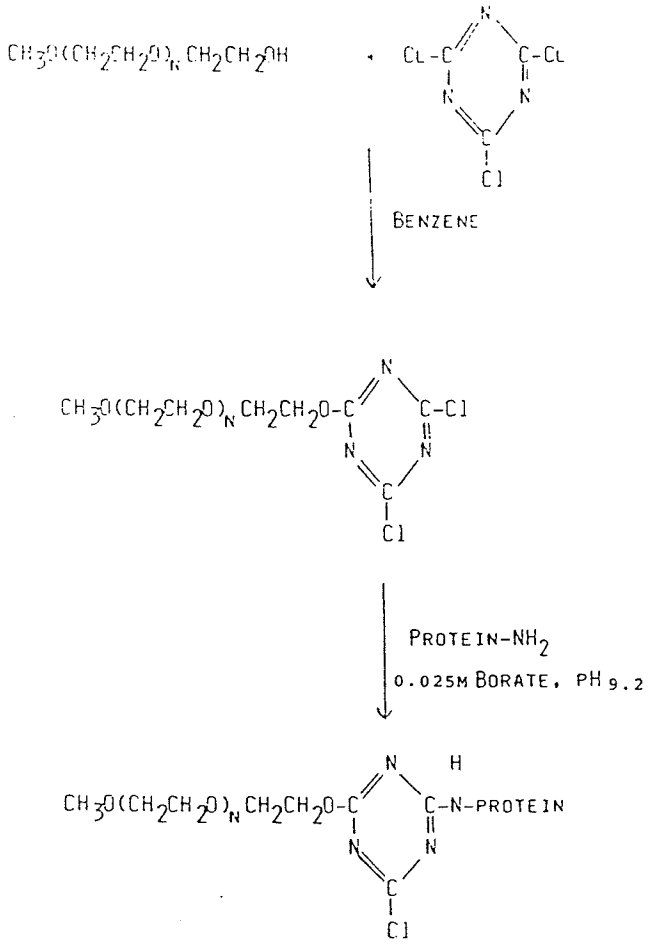
The mPEG (20g) was dissolved in 320 ml of anhydrous benzene at 80^0C and any residual water removed by distillation of the solution down to 50% of the original volume. The following steps were performed under a nitrogen atmosphere. Recrystallised cyanuric chloride (6.64 g) was added to the solution, followed by anhydrous potassium carbonate (4 g). The mixture was stirred for 15 hr at RT. The mixture was then filtered and the filtrate mixed with anhydrous petroleum ether (150 ml) to bring about precipitation of the mPEG intermediate The precipitate was separated from the reactants on a sintered glass funnel. The last two steps were repeated a total of seven times to remove all unreacted cyanuric chloride. The intermediate was dissolved in benzene, frozen at 78^0C and the benzene removed by vacuum induced sublimation. The intermediate was then stored in sealed, flame dried vials and stored at -60^0C.

Synthesis of XIgG(mPEG)$_n$ conjugates

Myeloma HIgG (10 mg/ml) in borate buffer pH = 9.6, purified as described above, was stirred with anhydrous (`activated') mPEG, of average molecular weight of 6,548, at 4^0C for 1 hr (171). The conjugation is illustrated schematically in figure 1. Anhydrous mPEG was reacted with HIgG#1 at molar ratios of 100:1, 44:1 and 18:1 and yielded conjugates with n values of 20, 13 and 5 respectively. HIgG#2 (6 mg/ml) was reacted with a different batch of anhydrous

Figure 1:

PREPARATION OF MONOMETHOXYPOLYETHYLENE GLYCOL CYANURIC CHLORIDE INTERMEDIATE AND THE SYNTHESIS OF m-PEG-PROTEIN CONJUGATES



intermediate which was less potent than that used for conjugation of HIgG#1, thus a molar ratio of 700:1 (mPEG to HIgG#2) yielded a conjugate with an n value of 17. Unconjugated mPEG was removed from the $HIgG(mPEG)_n$ by passage through an AcA-44 gel filtration column equilibrated in PBS and eluted with PBS (figure 2).

Sham conjugated (i.e., where n=0) was obtained by substituting hydrated (`deactivated') mPEG, at a molar ratio of 100:1, in the conjugation procedure for HIgG#1 and at 700:1 for HIgG#2. The position of the peak for sham conjugated HIgG is indicated by the arrow in figure 2.

The degree of substitution of the conjugates was determined as described previously.

Results

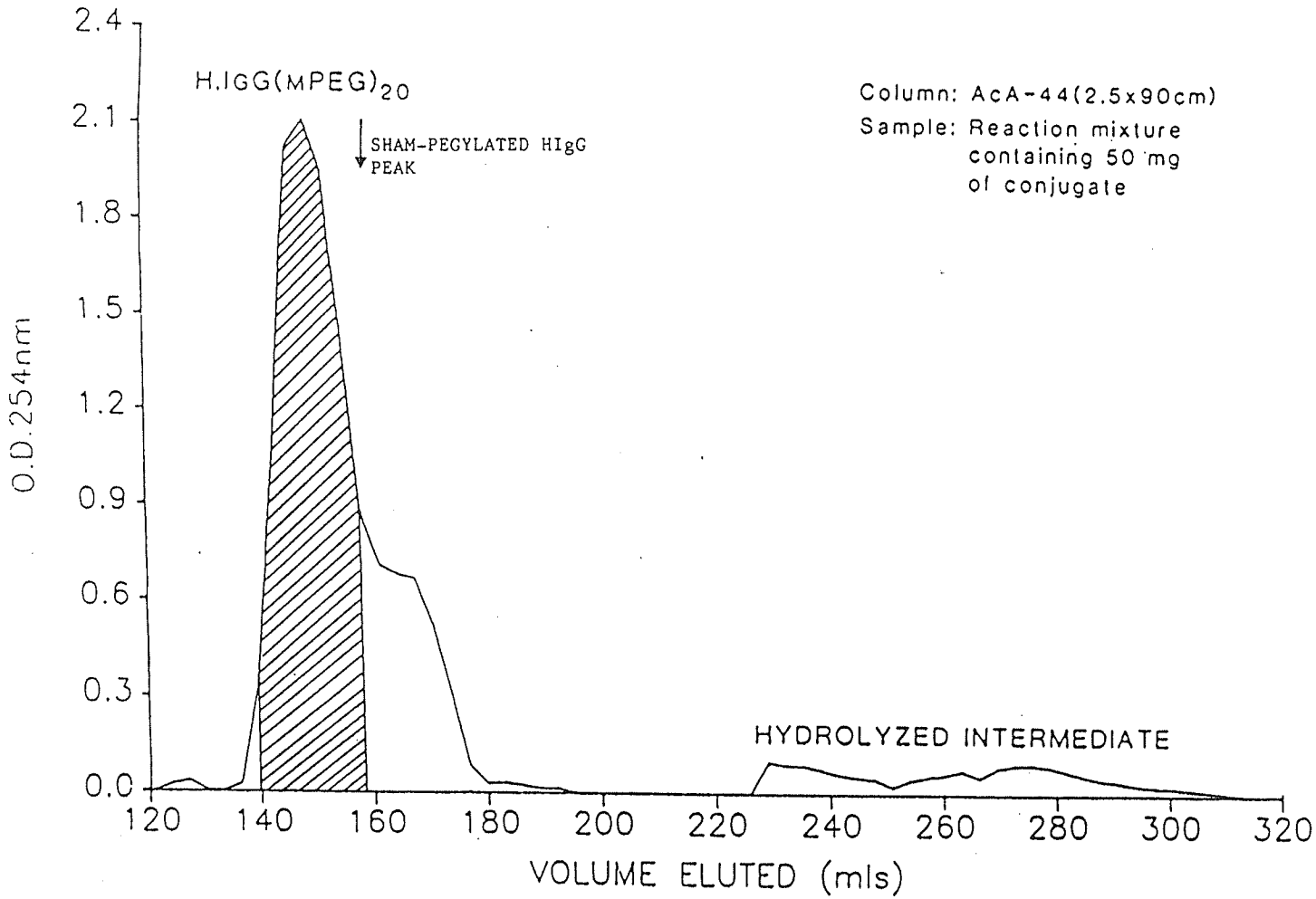
The separation of conjugate from hydrolysed intermediate and other products of the reaction is shown in figure 2. The fractions containing intermediate were identified using Nesslers' reagent. The conjugate peak itself was Nesslers' positive, followed by a number of Nesslers' negative fractions, then a number of Nesslers' positive fractions reflecting the elution of unconjugated hydrolysed intermediate. The presence or absence of mPEG in these three distinct zones was confirmed by NMR analysis.

Discussion

The method described here allowed the immunoglobulin to remain in solution throughout the conjugation and purification procedure, thus

Figure 2:

SEPARATION OF CONJUGATE FROM HYDROLISED INTERMEDIATE



reducing the possibility of aggregation. Passage through the AcA 44 column achieved separation of conjugate from hydrolysed intermediate and other products of the reaction with simultaneous exchange of buffers (borate pH = 9.6 into PBS pH = 7.4). Jackson et al (171) used a DEAE ion exchange column to separate hydrolysed intermediate from conjugates. The use of size exclusion gels in the present work to separate HIgG(mPEG)$_n$ conjugate from hydrolysed intermediate was found to be effective.

Tolerogenic properties of conjugates

Introduction

The tolerogenic capacity of the conjugates was tested in mice over a low dose range. Dresser (72) showed that adult CBA mice could be tolerised by large doses of BGG (70-300 mg). Chiller and Weigle (121) demonstrated that 100 micrograms of deaggregated HIgG would tolerise thymocytes whereas 2.5 mg was necessary to tolerise bone marrow cells. It was assumed initially that the HIgG(mPEG)$_n$ conjugates would behave in an analogous manner to deaggregated immunoglobulins and therefore, it was thought probable that a dose somewhere in the range of 1-100 micrograms would induce tolerance.

The route selected for administration of the conjugates was i.v. rather than i.p. This was based on the findings of Sulzberger (60) and Chase (61) that antigens administered systemically by the intravenous route tend to favour unresponsiveness rather than immunity.

The period of time (6 days) between the administration of tolerogen and immunisation was based on several considerations: Tolerance is complete in splenic B cells after approximately 3 days (124) and in splenic T cells in about 24 hours. However, bone marrow cells take 8-21 days to be tolerised (125). Loblay et al (172) demonstrated that maximal suppression was observed 7 days after administration of antigen in a study of the kinetics of suppressor cell generation using HGG. Parks and Weigle (127) showed that suppressor cell activity declined after the 10^{th} day following tolerance induction.

A subsequent study of the kinetics of tolerance induction to HIgG$(mPEG)_n$ conjugates indicated that the optimal time for induction of tolerance was between 6 and 43 days before immunisation.

Materials and Methods

Mice were injected with either 1, 10 or 100 micrograms of conjugate or PBS, i.v., and immunised 6 days later. Control groups were immunised with AOA. All groups were bled 14 and 21 days post-immunisation and their sera assayed for antibodies of the IgG1 class (i.e., the major murine isotype elicited by the immunisation protocol) with specificity for HIgG

Results

IgG1 titers are presented in table 1 and their means$^{\mp}$ for day 14 plotted in figure 3 (AOA control groups data excepted).

Considering the data presented in table 1(A); the greatest

$^{\mp}$geometric

Table 1-A: The effect of HIgG(mPEG)n conjugates on the primary IgG1 response to haIgG

		IgG1 anti-haIgG antibody titer			
conjugate n	treatment d -6 (µg)	mean d 14	S.D. d 14	mean d 21	S.D. d 21
-	PBS	18,133	1,644	16,833	1,132
20	1	15,333	3,055	14,500	2,211
"	10*	1,933	1,069	1,867	808
"	100*	1,000	600	867	416
13	1	17,767	1,124	16,400	1,422
"	10*	6,600	849	8,100	1,839
"	100*	6,867	4,580	4,500	1,852
5	1	18,200	1,908	16,533	2,444
"	10	12,467	3,937	9,733	3,001
"	100	7,850	1,202	8,113	1,848
0	1	19,467	2,501	18,033	3,661
"	10	15,500	1,609	14,567	3,004
"	100	13,833	1,756	13,400	1,769

Mice were injected with either 1, 10 or 100 micrograms of conjugate or PBS, i.v., and immunised with haIgG 10 micro -grams, i.p.) six days later. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Table 1-B: The effect of HIgG(mPEG)n conjugates on the primary IgG1 response to AOA.

		IgG1 anti-haIgG antibody titer			
conjugate n	treatment d -6 (μg)	mean d 14	S.D. d 14	mean d 21	S.D. d 21
-	PBS	8,467	736	9,837	801
20	100	7,813	445	9,423	1,182
13	"	8,507	278	9,570	1,339
5	"	9,867	1,725	9,623	935
0	"	8,957	1,157	10,530	1,953

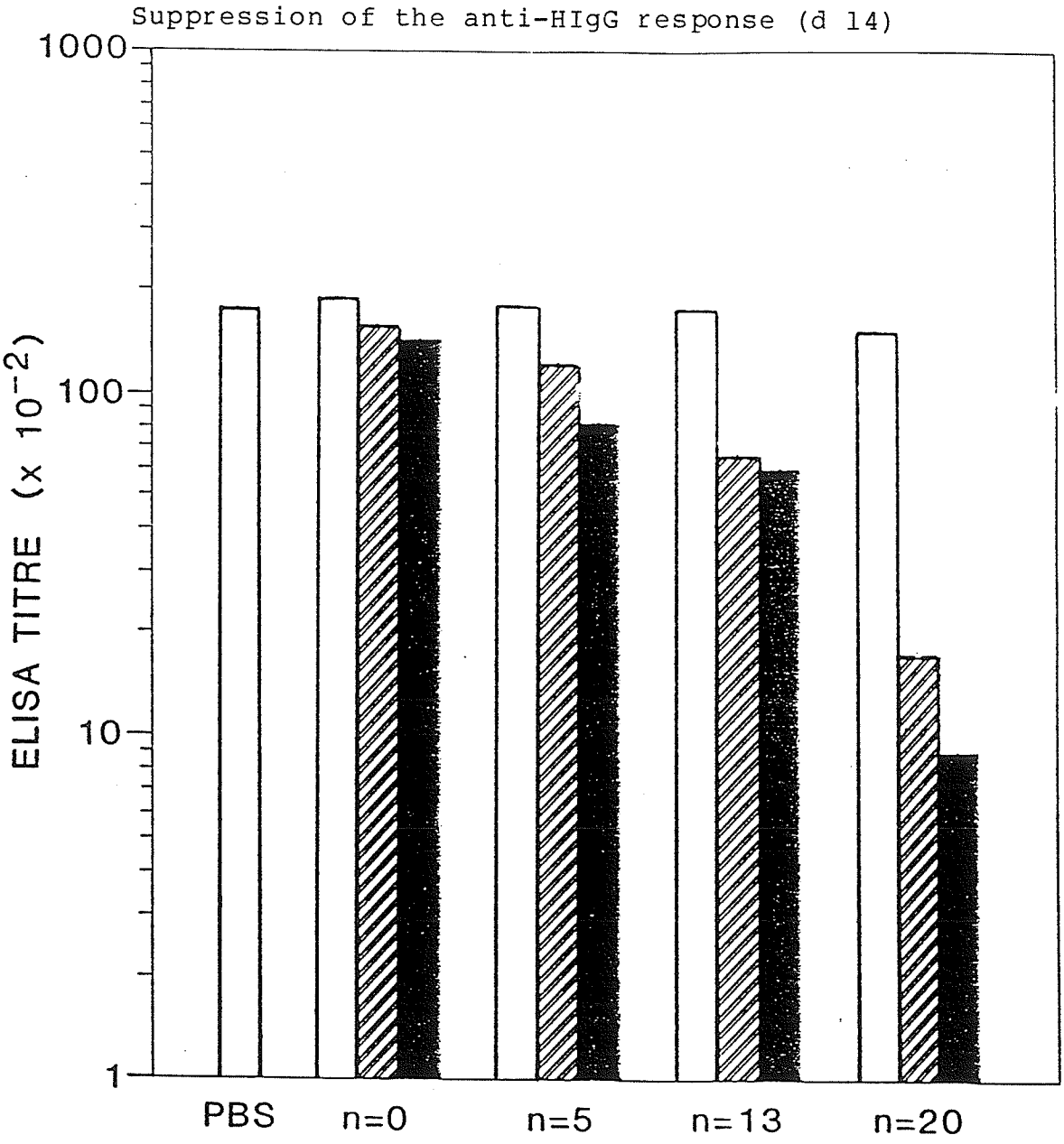
Mice were injected with either 1, 10 or 100 micrograms of conjugate or PBS, i.v., and immunised with AOA (50 micro-grams, i.p.) six days later. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-OA antibodies.

Figure 3: Legend
Suppression of the anti-HIgG response (d 14)

Values illustrated are for day 14 titers of IgG1 anti-HIgG antibodies and are the geometric means of 3 separate experiments. Open bars = 1 microgram of conjugate; stipled bars = 10 micrograms of conjugate; black bars = 100 micrograms of conjugate.

Groups of mice received one of the following, i.v., PBS, $HIgG(mPEG)_5$ (n=5), $HIgG(mPEG)_{13}$ (n=13), $HIgG(mPEG)_{20}$ (n=20) or sham-conjugated HIgG (n=0). These groups were immunised 6 days later with 10 micrograms of haIgG#1, i.p.. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Figure 3:


Suppression of the anti-HIgG response (d 14)
1000
100
10
1
ELISA TITRE (x 10^{-2})
PBS
n=0
n=5
n=13
n=20

48d

suppression was observed in recipients of 100 micrograms of $HIgG(mPEG)_{20}$ which had an average day 14 titer of 1,000 compared with 18,133 for recipients of PBS (95% suppression).

Recipients of 100 micrograms of the n=13, n=5 or n=0 conjugates showed average day 14 titers of 6,867, 7,850 and 13,833 respectively (i.e., 62%, 57% and 24% suppression respectively). Thus at a dose of 100 micrograms dose the amount of suppression induced was related to the degree of substitution, n.

Recipients of 10 micrograms of the n=20, n=13, n=5 and n=0 conjugates had average day 14 titers of 1,933, 6,600, 12,467 and 15,500 respectively (i.e., 89%, 64%, 31% and 15% suppression respectively). Thus at a dose of 10 micrograms the amount of suppression was also related to the degree of substitution, n.

Recipients of 1 micrograms of the n=20 conjugate had an average day 14 titer of 15,333 i.e., 16% suppression. The other conjugates had little or no suppressive effect at this dose.

No significant suppression was observed in groups treated with sham conjugated HIgG i.e., where n=0, or in groups treated with $HIgG(mPEG)_5$.

Thus the degree of suppression was related to the dose of conjugate administered and to the degree of conjugation. For all groups day 14 titers were not statistically different from the titers for day 21.

The suppression was shown to be specific (see table 1(B)), in that injection of 100 micrograms of $HIgG(mPEG)_n$ did not influence an

anti-ovalbumin (OA) IgG1 antibody response in mice immunised with AOA. For example mice treated with 100 micrograms of the n=20, n=13, n=5 or n=0 conjugates showed average day 14 titers of 7,813, 8,507, 9,867 and 8,957 respectively compared with 8,467 for mice given PBS in place of conjugate.

Discussion

The amount of suppression observed was related to the dose of conjugate administered and to n, the degree of substitution. The highest degree of tolerance was observed using 100 micrograms of $HIgG(mPEG)_{20}$ which was therefore selected for subsequent studies.

Dresser (78) showed that altering the physical state of gamma globulins rendered them either immunogenic or tolerogenic depending upon the degree of aggregation. It is possible that the conjugation procedure has the net effect of reducing the amount of aggregation, thereby conferring some suppressive potential upon the sham conjugated proteins. However, the conjugates per se are more potent tolerogens than their sham conjugated HIgG counterparts at 10 and 100 micrograms doses. Thus the covalent attachment of an appropriate number of mPEG molecules to HIgG results in conjugates possessing tolerogenic capacities greater than that induced when HIgG is subjected to the sham conjugation process.

These results are consistent with those of Lee and Sehon (154-156) (albeit for a different isotype) who showed that $OAm(PEG)_n$ conjugates were capable of suppressing the IgE response to OA in a murine model;

and with the results of Wie et al (163) using mPEG conjugates of OA, Timothy grass pollen, Dog albumin and bovine pancreatic ribonuclease to reduce the IgE response in sensitized animals. Similar findings were reported by King et al (164,165) using Ragweed antigen E-$(mPEG)_n$ conjugates.

Injection of an admixture of HIgG#1 and hydrolysed intermediate

Introduction

In order to examine the possibility that the presence of non-covalently bound intermediate was responsible for the induction of tolerance , an admixture of HIgG#1 and hydrolysed intermediate was administered to mice and its suppressive ability compared with recipients of HIgG$(mPEG)_{20}$ or of sham conjugated HIgG.

Materials and Method

One hundred micrograms of HIgG#1 was mixed together with either 8, 80 or 800 micrograms of hydrolysed (inactive) intermediate and given i.v. into naive mice (100 micrograms of HIgG$(mPEG)_{20}$ contains, on average, 8×10^{-5} g of mPEG; therefore this amount together with amounts 10 fold higher and 10 fold lower were injected into mice). Other groups received either PBS or PBS plus 100 micrograms of sham conjugated HIgG#1 or 8, 80 or 800 micrograms of intermediate plus PBS or 100 micrograms of HIgG$(mPEG)_{20}$. All groups were immunised 6 days later and bled 14 and 21 days post-immunisation.

Results

IgG1 titers are presented in table 2. Recipients of 100 micrograms of HIgG(mPEG)$_{20}$ had an average day 14 titer of 1,763 compared with 20,069 for recipients of PBS i.e., 91% suppression; and showed suppression compared with all other groups. By day 21 after immunisation, only the group treated with 100 micrograms of HIgG(mPEG)$_{20}$ showed significant suppression, i.e., the suppression in groups (a) and (d) on day 14 was not only slight but also transient.

Mice treated with 8, 80 or 800 micrograms of mPEG or with 8 or 800 micrograms of mPEG plus 100 micrograms of sham conjugated HIgG showed no suppression in comparison with mice that received PBS in place of conjugate. Mice treated with 80 micrograms of mPEG plus 100 micrograms of sham conjugated HIgG or mice treated with 100 micrograms of sham conjugated HIgG alone, showed some suppression when compared with mice receiving PBS but not to the same extent as mice receiving 100 micrograms of HIgG(mPEG)$_{20}$.

Recipients of an admixture of 80 micrograms of hydrolysed mPEG and 100 micrograms of sham conjugated HIgG had an average day 14 titer of 16,102 (20% suppression) which was similar to 15,165 (24% suppression) for recipients of 100 micrograms of sham conjugated HIgG alone. Thus the observed suppression was probably due to the presence of the sham conjugated HIgG which has been shown previously to induce some suppression.

Recipients of an admixture of 100 micrograms of sham conjugated HIgG with either 8 or 800 micrograms of hydrolysed mPEG showed

Table 2: Effect of an admixture of HIgG and mPEG on the IgG1 anti haIgG response

group	d -6	d 0	IgG1 titer d 14 mean	S.D.
a*	80 µg mPEG + 100 µg sham HIgG	haIgG	16,102	177
b	80 µg mPEG + PBS	"	20,323	1,288
c*	PBS + 100 µg sham HIgG	"	15,165	1,492
d	8 µg mPEG + 100 µg sham HIgG	"	16,322	1,242
e	8 µg mPEG + PBS	"	19,708	431
f	800 µg mPEG +100 µg sham HIgG	"	16,322	961
g	800 µg mPEG + PBS	"	21,382	1,667
h	PBS + PBS	"	20,069	1,512
i*	100 µg HIgG(mPEG)20	"	1,763	931

100 micrograms of HIgG#1 was mixed together with either 8, 80 or 800 micrograms of hydrolysed intermediate and given i.v. into naive mice. Other groups received either PBS or PBS plus 100 micrograms of sham conjugated HIgG#1 or 8, 80 or 800 micrograms of intermediate plus PBS or 100 micrograms of HIgG(mPEG)20. All groups were immunised 6 days later with 10 micrograms of haIgG, i.p., and bled 14 and 21 days post -immunisation.

Table 2-continued: Effect of an admixture of HIgG and mPEG on IgG1 anti-haIgG response

group	d -6	d 0	IgG1 titer d 21 mean	S.D.
a	80 µg mPEG + 100 µg sham HIgG	haIgG	15,790	1,085
b	80 µg mPEG + PBS	"	20,233	1,587
c	PBS + 100 µg sham HIgG	"	17,000	25
d	8 µg mPEG + 100 µg sham HIgG	"	17,906	876
e	8 µg mPEG + PBS	"	19,931	1,935
f	800 µg mPEG +100 µg sham HIgG	"	15,613	568
g	800 µg mPEG + PBS	"	20,655	1,730
h	PBS + PBS	"	20,441	2,891
i	100 µg HIgG(mPEG)20	"	2,471	721

100 micrograms of HIgG#1 was mixed together with either 8, 80 or 800 micrograms of hydrolysed intermediate and given i.v. into naive mice. Other groups received either PBS or PBS plus 100 micrograms of sham conjugated HIgG#1 or 8, 80 or 800 micrograms of intermediate plus PBS or 100 micrograms of HIgG(mPEG)20. All groups were immunised 6 days later with 10 micrograms of haIgG, i.p., and bled 14 and 21 days post-immunisation.

similar amounts of suppression (19% for both) to that for an admixture of 100 micrograms of sham conjugated HIgG with 80 micrograms of hydrolysed mPEG (20%).

Discussion

The administration of sham conjugated HIgG admixed with hydrolysed mPEG, resulted in some suppression compared with mice receiving PBS. However, this suppression is of the same order as that observed sham conjugated HIgG and much less than that observed when mice are treated with the conjugates per se. A 100 fold change in the amount of hydrolysed mPEG present in the admixture does not significantly effect the observed suppression indicating that an admixture of hydrolysed mPEG with sham conjugated protein is not immunologically equivalent to the covalently bound conjugate i.e., there is a requirement that the mPEG be bound to the protein in order that the suppression be induced.

In vivo fate of ^{125}I-HIgG(mPEG)$_{20}$ and ^{125}I-sham conjugated HIgG#1

Introduction

The in vivo fates of radiolabelled derivatives of HIgG(mPEG)$_{20}$ and of sham conjugated HIgG were studied in order to discover (a) if the attachment of mPEG to HIgG altered the latter's clearance rate and (b) whether or not the conjugate would be accumulated in certain organs.

Materials and Method

HIgG(mPEG)$_{20}$ and sham conjugated HIgG#1 were radioiodinated by the

chloramine T method (170) as described previously. For radiolabelled HIgG(mPEG)$_{20}$ there was on average one iodine atom per 637 molecules of the conjugate. For radiolabelled sham conjugated HIgG there was on average one iodine atom per 508 molecules of protein. These substances were then diluted with cold protein or conjugate. One hundred micrograms (1×10^7 cpm) of either protein were injected, i.v. into mice that were maintained on KI drinking water for 5 days prior to, and throughout the experiment.

Commencing 24 hr after administration of the proteins and every 24 hr thereafter for 8 days, groups of mice were bled into disposable test tubes and 100 microlitres of blood transferred into plastic counting tubes. The mice were then killed and the following organs excised for counting: spleen, kidneys, thymus, liver and mesenteric lymph nodes (MLN). All organs and 100 microlitres samples of blood were counted for 5 min and cpm recorded.

Results

The percent cpm remaining for blood and organs over the 8 day period of the experiment are presented in table 3. The data for clearance from the blood are plotted in figure 4. All values are minus background. Values for blood are corrected for total blood volume i.e., assuming a total blood volume of 2.5 ml in adult mice; therefore the original data which was cpm in 100 microlitres has been multiplied by 25.

Considering the clearance of the radiolabelled conjugate and the

Table 3: The in vivo fate of radiolabelled sham conjugated HIgG

	Mean (2 data points per mean) percent cpm remaining (1×10^7 cpm = 100%)					
Day	Thymus	Liver	Kidneys	Spleen	MLN	Blood
1	0.093	1.755	0.565	0.108	0.020	11.250
2	0.025	1.030	0.335	0.070	0.057	6.230
3	0.027	0.800	0.290	0.055	0.050	3.960
4	0.026	0.545	0.220	0.031	0.015	3.720
5	0.011	0.340	0.185	0.033	0.048	2.545
6	0.011	0.335	0.160	0.024	0.027	2.155
7	0.008	0.275	0.110	0.019	0.016	1.410
8	0.006	0.220	0.085	0.017	0.014	1.070

Sham conjugated HIgG#1 was radiolabelled then diluted with cold HIgG#1 100 micrograms of this mixture containing 1×10^7 cpm was injected i.v into mice that were maintained on KI drinking water for 5 days prior and throughout the experiment. Commencing 24 hours after administrat -ion of the protein and every 24 hours thereafter (for 8 days in total) groups of mice were bled and then killed. The organs listed in the table above were excised and counted. Values are expressed as mean percent cpm remaining.

Table 3-B: The in vivo fate of radiolabelled HIgG(mPEG)20

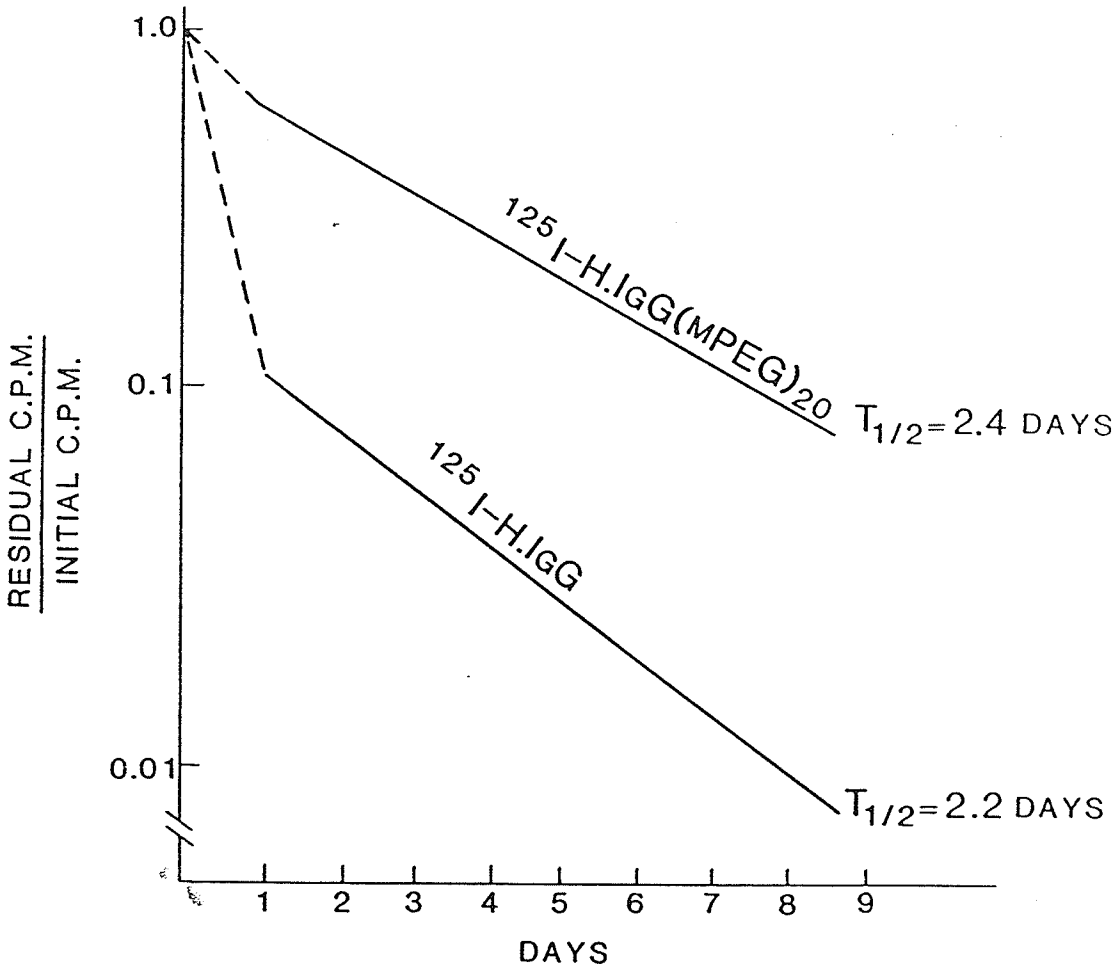
	Mean (2 data points per mean) percent cpm remaining (1×10^7 cpm = 100%)					
Day	Thymus	Liver	Kidneys	Spleen	MLN	Blood
1	0.120	4.130	1.445	0.360	0.085	55.710
2	0.135	3.260	1.125	0.290	0.067	40.880
3	0.115	2.405	0.770	0.170	0.210	31.295
4	0.070	2.205	0.695	0.180	0.170	28.395
5	0.060	1.495	0.495	0.098	0.100	20.270
6	0.030	0.900	0.330	0.069	0.050	14.185
7	0.028	1.110	0.200	0.305	0.070	6.675
8	0.006	0.320	0.420	0.240	0.103	6.300

HIgG(mPEG)20 was radiolabelled then diluted with cold conjugate 100 micrograms of this mixture containing 1×10^7 cpm was injected i.v into mice that were maintained on KI drinking water for 5 days prior and throughout the experiment. Commencing 24 hours after administration of the protein and every 24 hours thereafter (for 8 days in total) groups of mice were bled and then killed. The organs listed in the table above were excised and counted. Values are expressed as mean percent cpm remaining.

Figure 4: Legend
Clearance of i.v. administered ^{125}I-labelled HIgG and HIgG(mPEG)$_{20}$ from circulation of normal B6D2F1 mice after 24 hours equilibration.

HIgG(mPEG)$_{20}$ and sham conjugated HIgG#1 were radioiodinated. diluted with cold protein and 100 micrograms injected (1×10^7 cpm) i.v. into mice that were maintained on KI drinking water for 5 days before, as well as during the experiment. Begining 24 hours after administration groups of mice were bled and radioactivity counted. These counts are plotted as the ratio: residual cpm/initial cpm versus time (days).

Figure 4: CLEARANCE OF INTRAVENOUSLY ADMINISTERED ^{125}I-LABELLED H.IgG AND H.IgG(MPEG)$_{20}$ FROM CIRCULATION OF NORMAL B6D2F1 MICE AFTER 24 HOURS EQUILIBRATION



1. RELATIVE BLOOD LEVEL OF H.IgG-MPEG$_{20}$ SIX TIMES HIGHER THAN THAT OF H.IgG.

2. ESSENTIALLY SIMILAR HALF-LIVES WERE OBTAINED FOR CLEARANCE FROM LIVERS, SPLEENS, KIDNEYS AND THYMUSES; NO EVIDENCE FOR PREFERENTIAL LOCALIZATION. BLOOD LEVELS ABOUT 12-FOLD HIGHER THAN CUMULATIVE LEVELS OF ALL ORGANS EXAMINED.

sham conjugated control, the relative blood levels of $HIgG(mPEG)_{20}$ are approximately six times greater than those of the sham conjugated control; but after the first 24 hours their half-lives are equivalent (2.4 and 2.2 days respectively). The two clearance curves, although parallel, are distinct, having different intercepts. The levels of radiolabel in the blood were approximately 12 fold higher than the cumulative levels of all organs examined.

Considering now the data (table 3) for clearance from organs, essentially similar half-lives were obtained for clearance from liver, spleen, kidneys and thymus. The greatest number of counts were obtained from the liver but this is also a large, highly vascularized organ. Levels of radioactivity remaining in the organs studied were small in comparison with levels in the blood. The data obtained for the mesenteric lymph nodes (MLN) were not consistent. This may reflect the somewhat amorphous nature of these nodes in comparison with, for example, a kidney with consequent problems encountered in their complete excision.

Discussion

The possibility that the amount of radioactivity detected in recipients does not necessarily reflect the amount of tolerogen must be considered. It is possible that the radiolablel was excised from the rest of the tolerogen (or derivative thereof) in vivo and that there is no correlation between cpm and amount of conjugate present. In addition it is possible that the radioiodination of conjugate

molecules may not occur with equal probability, i.e., in a conjugate designated as HIgG(mPEG)$_n$ there will in reality be a distribution of degrees of substitution about N. If this is so, then the radiolabelled conjugate molecules may not be representative of the whole population. These limitations of the method must be born in mind when interpreting data derived from experiments of this type.

The above data show that the attachment of mPEG to HIgG results in blood levels 6 times greater than that of the native protein. Induction of tolerance by conjugate may be related to either a decreased amount of phagocytosable (immunogenic) material or to an increased amount of tolerogen remaining in vivo, in comparison with the sham-conjugated HIgG. This effective increase in the dose of tolerogen over time compared with the effective dose of native protein over the same period may be significant for the induction and maintenance of tolerance (66,68,70,74). The initial rapid elimination over the first 24 hr period may reflect the clearance of phagocytosable (aggregated) protein (120). HIgG(mPEG)$_n$ conjugates have been shown to contain less aggregated material than the native protein (167) and therefore after 24 hours there is more HIgG(mPEG)$_{20}$ remaining since it contained less aggregated material than was present in the sham conjugated protein. [The effect of the physical state of the tolerogen and the possible role of mPEG in tolerance induction are discussed in detail in chapter V, General Discussion].

Davis et al (150), using PEG conjugates of uricase, catalase, arginase, asparaginase and glutaminase showed that the conjugates had

longer in vivo half-lives than the unmodified native enzymes. However, there is clearly some difference on the in vivo fates of these enzymes and the fate of HIgG because all the native enzymes had very short half-lives (e.g., < 6 hours for arginase and catalase) compared with the relatively long half-life of native HIgG (2-3 days). The attachment of twenty mPEG molecules to HIgG does not appear to alter the latter's rate of catabolism. This would imply that recognition of xenogeneic immunoglobulin by phagocytes and enzymatic degradation is not affected by the presence of mPEG groups on the immunoglobulin. Presumably mPEG groups do not sterically hinder macrophage receptor interactions with the conjugate nor conjugate/catabolic enzyme interactions.

Similar findings were reported by Lee and Sehon (155) who showed that OA-$(PEG)_6$ conjugate was present at 10 times the level of OA after 24 hr in vivo

Abuchowski et al (148) have reported that conjugates of PEG with the enzymes uricase, arginase or catalase do not accumulate in organs or tissues, or show evidence of damage to the animal.

Kinetics of tolerance induction by HIgG(mPEG)$_{20}$

Introduction

The amount of time required to induce tolerance in vivo varies markedly depending upon the antigen, dose and the cell population(s) under consideration. In general, both peripheral and thymic T lymphocytes become tolerant rapidly (56). The B cell population

tends to become tolerant more slowly than does the T cell population (127). The splenic B cell population becomes tolerant more rapidly than the bone marrow B cell population (126).

In order to investigate the time required to induce tolerance the interval between administration of conjugate and immunization was varied. [The kinetics of transferable tolerance are discussed in chapter 3]. In addition the effect of administration of conjugate one or two days after immunization was studied.

Materials and Method

Groups of mice were given 100 micrograms of $HIgG(mPEG)_{20}$, i.v., and immunised either 6 hours, 1, 6, 15, 29, 43, 57 or 63 days later. Other groups were immunised and then given conjugate either one or two days later. In addition one group of mice received conjugate and immunogen almost simultaneously (tolerogen one or two minutes prior to immunization). Control groups received PBS in place of conjugate. All groups were bled 14 and 21 days post-immunization.

Results

IgG1 titers are presented in table 4 [Please note that this table is reproduced in part in chapter 3 under the section dealing with comparison of the duration of transferable tolerance with that of tolerance in the intact mouse; and appears there for the purpose of comparison]. The percent suppression for day 14 mean titers are plotted in figure 5.

Table 4:
The effect of varying the interval between tolerising and immunising injections on the suppressive ability of HIgG(mPEG)20

	Mean IgG1 titer (2 data points per mean)			
History	day 14	S.D.	day 21	S.D.
haIgG,Tol d2*	30,260	2,452	30,521	1,686
haIgG,Tol d1	18,239	1,832	18,645	2,786
Tol,haIgG	12,544	1,526	14,505	1,718
Tol,haIgG 6 hr	9,240	382	10,255	403
Tol,haIgG d1*	5,593	465	8,474	1,547
Tol,haIgG d6*	3,100	424	3,200	849
Tol,haIgG d15*	2,804	448	3,163	355
Tol,haIgG d29*	4,066	1,486	4,285	729
Tol,haIgG d43*	3,310	1,582	4,466	1,069
Tol,haIgG d57*	8,326	839	7,351	806
Tol,haIgG d63	18,022	36	18,864	1,131
PBS,haIgG	16,365	1,677	17,620	1,867

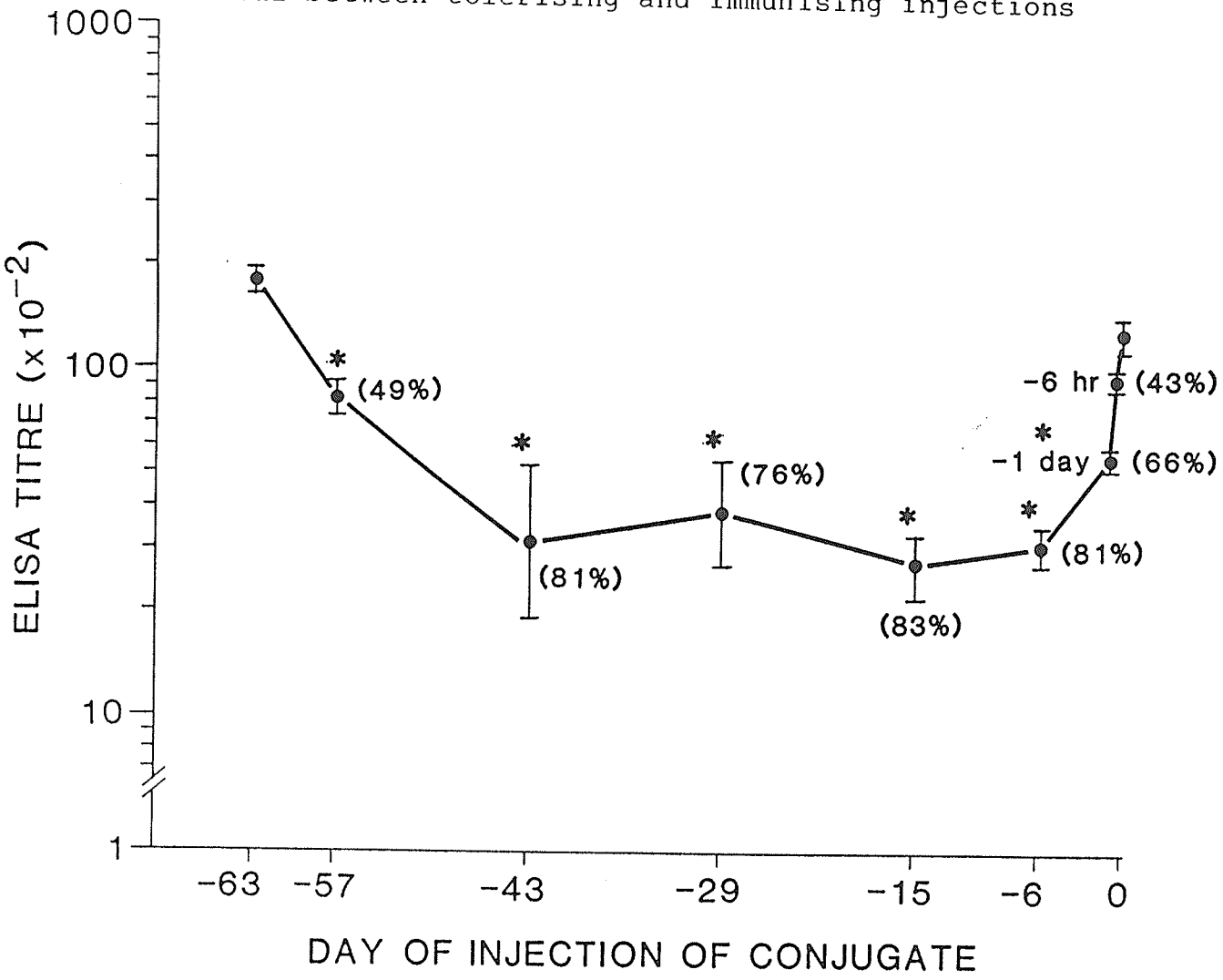
Groups of mice were given 100 micrograms of HIgG(mPEG)20 i.v., and immunised with 10 micrograms of haIgG, i.p., either 6 hours, 1,6,15,29,57 or 63 days later. Other groups were immunised and then given conjugate either one or two days later. In addition one group of mice received conjug -ate and immunogen simultaneously. Control groups received PBS in place of conjugate. All groups were bled 14 and 21 days post-immunisation.

Figure 5: Legend
Percent suppression vs interval between tolerising and immunising injections.

Groups of mice received 100 micrograms of HIgG(mPEG)$_{20}$, iv. and were then immunised with 10 micrograms of haIgG i.p. 6 hours, 1, 6, 15, 29, 57 or 63 days later. Other groups were immunised and then given conjugate either one or two days later. In addition one group of mice received conjugate and immunogen simultaneously. Control groups received PBS in place of conjugate. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Immunisation day = 0. Figures in parentheses are percent suppression, taking the group that received PBS in place of conjugate on day -6 as 100% response. The "T-bars" represent the standard error of the mean.

Figure: 5 Percent suppression
vs interval between tolerising and immunising injections



Administration of conjugate 6 to 43 days prior to immunisation resulted in approximately 80% suppression. Suppression was lower (although still significant) (66%) when the conjugate was administered 1 day prior to immunisation.

No significant effect was observed when the conjugate was injected one day after immunisation, immediately before, 6 hr or 63 days prior to immunisation.

Mice treated with conjugate 6, 15, 29 or 43 days prior to immunsation had average day 14 titers of 3, 100, 2,804, 4,066 and 3,310 respectively compared with 16,365 for mice given PBS in place of conjugate. Mice treated with conjugate one day prior to immunisation showed less suppression than that observed for the mentioned groups with average day 14 titers of 5,593 and 9,240 respectively. Mice treated with conjugate one or two days after immunisation showed no suppression with average day 14 titers of 18,339 and 30,260 respectively.

Discussion

Suppression, ranging from 76% to 83% was observed when the tolerogen was administered from 6 to 43 days prior to immunisation. In contrast some enhancement of the immune response was observed when the conjugate was injected 2 days after immunisation. No significant suppression was observed if the conjugate was given simultaneously with, or 6 hrs of 63 days prior to immunisation.

The antibody response to haIgG is T cell dependent and therefore

requires helper T cell function (119). In the HGG system, tolerance in splenic T cells is complete within 24 hrs of injection of tolerogen (127). Thus it would appear that the reduced suppression observed in groups immunised within 1 day of administration of $HIgG(mPEG)_{20}$ was not due simply to insufficient time for induction of tolerance.

Considering the other end of the time scale, Chiller et al (125) have demonstrated complete unresponsiveness in thymocytes for 120 days, following administration of milligram doses of deaggregated HGG. The lower dose (100 micrograms) of conjugated HIgG used in the present work induced tolerance for a maximum of approximately 43 days.

PART B: Synthesis and properties of $HIgG(mPEG)_{17}$

Induction of tolerance by $HIgG(mPEG)_{17}$ and comparison of i.v. and i.p. routes of tolerogen administration

Introduction

In order to test whether or not the tolerogenic properties of the HIgG#1 derived conjugates were unique to that myeloma, a second myeloma (HIgG#2) was purified and conjugated by the same method as was described above for the synthesis of the HIgG#1 conjugates but using a different batch of intermediate. As the n=20, HIgG#1 conjugate had shown the greatest tolerogenicity, an attempt was made

to synthesise a conjugate of HIgG#2 with an even greater degree of substitution. However, the conjugate obtained was found to have an average degree of substitution of 17. This may be due to the fact that the batch of intermediate used in the synthesis of $HIgG(mPEG)_{17}$ was less potent than the batch used to make the HIgG#1 series of conjugates. Work by Dr C-J. C. Jackson (unpublished observations) involving the synthesis of $OA(mPEG)_n$ conjugates utilising the same batch of intermediate also showed reduced potency resulting in lower n values than predicted for $OA(mPEG)_n$ conjugates.

Materials and Methods

The in vivo tolerogenic properties of $HIgG(mPEG)_{17}$ were determined using the same protocol as described above, except that some mice received i.p. injections of conjugate. An aliquot of the HIgG#2 myeloma was heat-aggregated for use as an immunogen by the same method as described for HIgG#1. Fifty micrograms of haIgG#2 , i.p. were used for immunisation compared with 10 micrograms of haIgG#1. This was in order to elicit IgG titers in the same range (20-27,000) as were obtained with haIgG#1. The difference in the `immunising potency' of the two myelomas (i.e., 10 micrograms compared with 50 micrograms) may reflect differences in the ease of aggregation of the two proteins. Similar variations were observed in earlier pilot studies using various batches of commercial (Capell) bovine IgG. For all experiments involving $HIgG(mPEG)_{17}$ total IgG was determined by ELISA.

Sham conjugation of HIgG#2 was also performed using hydrolysed intermediate (700:1 molar ratio, mPEG to protein) and given in place of conjugate in control groups. Other control groups were immunised with AOA in place of haIgG.

Results

IgG titers are presented in table 5 and the means are plotted in figure 6. (Data for AOA control groups excepted). Considering table 5; Mice treated either i.v. or i.p. with either 10 or 100 micrograms of HIgG(mPEG)$_{17}$ showed suppression compared with mice receiving PBS. At a dose of 10 micrograms the i.v. route was superior to the i.p. route with respect to induction of tolerance. No difference was observed between i.p. and i.v. routes of administration at a dose of 100 micrograms.

Administration of 10 or 100 micrograms of sham conjugated HIgG resulted in some suppression compared with recipients of PBS; The suppression induced by 100 micrograms of sham conjugated HIgG was not as great as that induce by the same dose of HIgG(mPEG)$_{17}$.

Administration of 100 micrograms of HIgG(mPEG)$_{17}$ either i.v. or i.p. had no effect on the anti-AOA response.

One hundred micrograms of HIgG(mPEG)$_{17}$ given i.v. induced the greatest suppression (84%) of IgG titers with an average day 14 titer of 3,826 compared with 23,993 for mice receiving PBS. Recipients of the same dose but administered by the i.p. route, showed an average day 14 suppression of 69%. Recipients of 100 micrograms of the sham

Table 5: In vivo testing of HIgG(mPEG)17

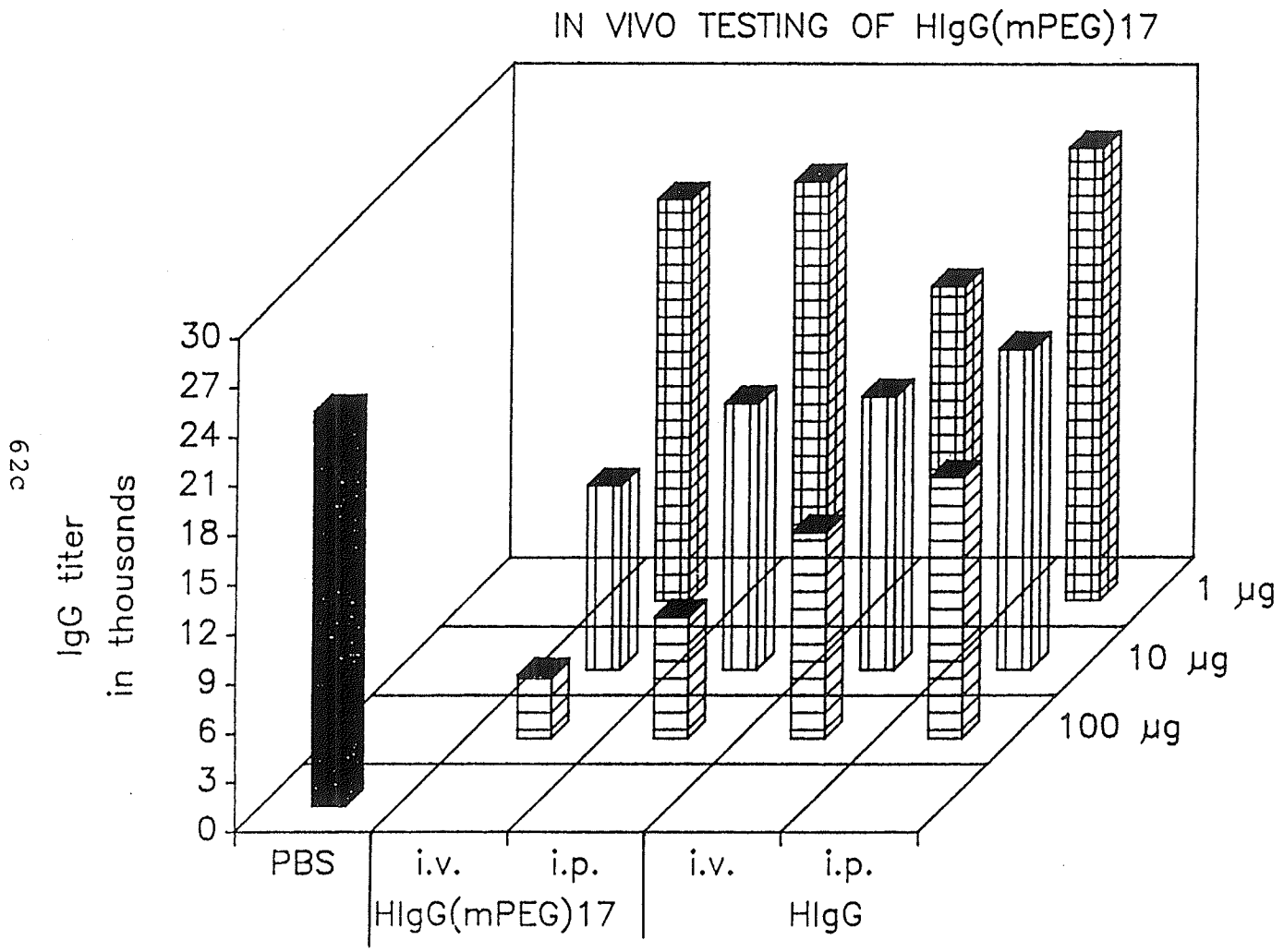
group	d -6		route	d 0	mean IgG titer (2 data points) day 14	S.D.
a	PBS		i.v.	haIgG	23,993	2,816
b	1 µg HIgG(mPEG)17		"	"	24,288	1,792
c*	10		"	"	11,149	2,241
d*	100		"	"	3,826	1,059
e*	" "	"	"	AOA	16,589	1,849
f	1		i.p.	haIgG	25,383	1,642
g*	10		"	"	16,203	2,175
h*	100		"	"	7,356	1,857
i*	" "	"	"	AOA	15,293	2,075
j	1	HIgG	i.v.	haIgG	19,031	2,150
k*	10		"	"	16,620	537
l*	100		"	"	13,697	4,514
m	" "	"	"	AOA	16,169	1,922
n	1		i.p.	haIgG	24,555	-
o*	10		"	"	19,243	4,576
p*	100		"	"	16,716	873
q*	" "	"	"	AOA	16,803	145
r	PBS	-	i.v.	"	15,755	1,067

Groups of mice received 1, 10 or 100 micrograms of HIgG(mPEG)17 or sham conjugated HIgG#2 or PBS, i.v. or i.p. and were immunized with 50 micrograms of haIgG#2 or 50 micrograms of AOA six days later. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-hIgG#2 antibodies, except for groups immunised with AOA which were assayed for IgG anti -OA antibodies.

Figure 6: Legend
In vivo testing of $HIgG(mPEG)_{17}$

Groups of mice received 1, 10 or 100 micrograms of $HIgG(mPEG)_{17}$, sham conjugated HIgG#2 or PBS, i.v. or i.p. and were immunised with 50 micrograms of haIgG#2 or 50 micrograms of AOA six days later. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies. Plot shows d14 titers.

Figure 6:


IN VIVO TESTING OF HIgG(mPEG)17
IgG titer
in thousands
30
27
24
21
18
15
12
9
6
3
0
1 µg
10 µg
100 µg
PBS
i.v.
i.p.
HIgG(mPEG)17
i.v.
i.p.
HIgG

conjugated control showed 48% suppression by the i.v. route and 34% suppression by the i.p. route.

Recipients of a 10 micrograms dose of conjugate had average day 14 titers of 11,149 (53% suppression) for i.v. administration of conjugate and 16,203 (33% suppression) for i.p. administration. Recipients of i.v. administered sham conjugated HIgG showed an average of 31% suppression. Recipients of i.p. administered sham conjugated HIgG showed 19% suppression.

Recipients of 1 micrograms of conjugate or sham conjugated conjugate showed no suppression by either route of administration.

Recipients of conjugate that were immunised with AOA in place of HaIgG showed no suppression of the anti-OA response (table 5); for example, mice treated with conjugate i.p. or i.v. had average day 14 titers of 16,589 and 15,293 respectively compared with 15,495 for mice given PBS in place of conjugate.

Discussion

$HIgG(mPEG)_{17}$ is capable of inducing specific suppression in mice at doses of 10 or 100 micrograms both by the i.v. and i.p. routes. Although i.v. administration of conjugate induces greater suppression than that induced by i.p. administration, the latter route may be preferred in protocols requiring multiple administrations of conjugate due to the stress experienced by the mice during the i.v. injection procedure. The i.p. route for the administration of tolerogen has been used by various workers: Golub and Weigle (120)

showed that as little as 50 micrograms of DHGG could induce tolerance in C57BL/6 mice when given i.p. Benjamin (141) showed that A/J mice could be tolerised with 100 micrograms of DHGG given i.p..

The effect of multiple injections of $HIgG(mPEG)_{17}$ on induction of tolerance to haIgG

Introduction

The primary application of tolerogenic derivatives of HIgG will be for the pretreatment of patients who will be receiving xenogeneic MAbs as part of their therapy. In the majority of cases this will involve multiple injections of MAbs. In order to simulate more closely a clinical regimen, groups of mice were given multiple immunisations and varying numbers of tolerising injections of $HIgG(mPEG)_{17}$. The induction of unresponsiveness to HIgG is dose dependent (120). Dresser (79) observed that mice receiving multiple tolerising injections of deaggregated BGG exhibited greater suppression than mice receiving single injections of BGG. Based on these observations it was postulated that multiple injections of $HIgG(mPEG)_{17}$ would cause suppression, despite daily immunising stimuli.

The effect of treatment was followed by ELISA of anti-HIgG IgG levels.

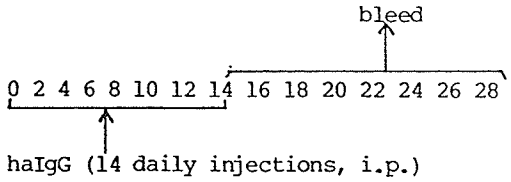
Materials and Methods

The experimental protocol is summarised in table 6. All mice

Table 6:

The effect of multiple injections of HIgG(mPEG)$_{17}$ upon anti-HIgG antibody titers

Protocol



Group	HIgG(mPEG)$_{17}$	Treatment
a	10 µg	-6 hrs. + 14 injections
b	100 "	" " "
c	10 "	" + 7 "
d	100 "	" " "
e	10 "	" + 5 "
f	100 "	" " "
g	PBS	" + 14 "

received 14 daily injections of 50 micrograms of haIg#2, i.p.. Groups of mice received either 10 microgram or 100 microgram doses of the conjugate, either daily (total = 15 injections), every second day (total = 8 injections) or every third day (total = 6 injections) for a period of 14 days. All these groups received their first injection of conjugate 6 hours before the first immunisation with haIgG#2. A control group received PBS instead of conjugate. All groups were bled on the 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 15^{th} and 22^{nd} day after the last injection of conjugate (i.e., on the 15^{th}, 17^{th}, 19^{th}, 21^{st}, 29^{th} and 36^{th} day of the experiment.).

Results

IgG titers are presented in table 7 and the means of the day 29 titers are plotted in figure 7.

IgM day 15 and day 29 titers for recipients of 100 micrograms doses of conjugate are presented in table 8 together with titers for mice receiving 100 micrograms of conjugate or PBS, for purposes of comparison.

Considering the IgG ELISA data; table 7 shows that treatment of mice with either 5,7 or 14 daily i.v. injections of either 10 or 100 micrograms of $HIgG(mPEG)_{17}$ resulted in suppression compared with mice receiving PBS. One hundred micrograms of conjugate was more effective than 10 micrograms for mice receiving either 14 or 5 daily injections. Mice receiving 14 injections at either 10 or 100 micrograms doses showed greater suppression than those receiving

Table 7:
The effect of multiple injections of HIgG(mPEG)17 upon the induction of tolerance to haIgG

treatment	Mean IgG titer (2 data points)			
µg	day 15	S.D.	day 17	S.D.
(a)* 10 -6 hr + 14 daily i.v.	73,403	3,195	76,665	2,832
(b)* 100 " "	17,069	1,944	20,822	4,005
(c)* 10 " + 7 daily i.v.	123,281	3,843	115,922	17,003
(d) 100 " "	61,193	1,906	58,500	5,735
(e)* 10 " + 5 daily i.v.	152,492	10,575	161,506	3,532
(f)* 100 " "	127,528	4,988	127,873	4,739
(g) PBS -6+ 14 daily i.v.	181,202	1,926	185,193	4,454
µg	day 19	S.D.	day 21	S.D.
(a)* 10 -6 hr + 14 daily i.v.	70,171	228	66,759	2,478
(b)* 100 " "	17,968	116	16,705	713
(c)* 10 " + 7 daily i.v.	124,710	4,453	117,005	5,657
(d)* 100 " "	63,556	5,358	58,378	2,118
(e)* 10 " + 5 daily i.v.	161,045	1,646	164,782	2,649
(f)* 100 " "	127,018	39	126,471	11,932
(g) PBS -6hr + 14 daily i.v.	181,132	4,368	177,771	2,039
µg	day 29	S.D.	day 36	S.D.
(a)* 10 -6 hr + 14 daily i.v.	62,558	2,352	56,889	1,747
(b)* 100 " "	18,333	1,879	21,356	-
(c)* 10 " + 7 daily i.v.	93,802	5,012	85,398	3,050
(d) 100 " "	64,090	-	66,008	-
(e) 10 " + 5 daily i.v.	152,397	6,360	139,953	5,740
(f)* 100 " "	118,549	11,788	97,736	4,948
(g) PBS -6hr + 14 daily i.v.	151,395	5,399	148,776	2,655

The experimental protocol is summarised in table 6. All mice recei -ved 14 daily injections of 50 micrograms of haIgG#2, i.p.. Groups of mice received either 10 or 100 microgram doses of conj -ugate daily (total = 15 injections), every second day (total = 8) or every third day (total = 6) for a period of 14 days. All groups received their first conjugate injection 6 hours before their first immunisation. A control group received PBS instead of con -jugate. All groups were bled on the 1st, 3rd, 5th, 7th, 15th and 22nd day following their last injection of conjugate. All sera were assayed for IgG anti-HIgG#2 antibodies.

Figure 7: Legend
Effect of multiple injections of HIgG(mPEG)$_{17}$ on mice receiving 14 daily injections of haIgG

The experimental protocol is summarised in table 6. All mice received 14 daily injections of 50 micrograms of haIgG#2, i.p.. Groups of mice received either 10 or 100 micrograms doses of conjugate daily (total = 15 injections) every second day) total = 8 injections) or every third day (total = 6 injections) for a period of 14 days. All groups received their first injection of conjugate 6 hours prior to their first immunisation. A control group received PBS in place of conjugate. All groups were bled on the 1st, 3rd, 5th, 7th, 15th and 22nd days following their last injection of conjugate. (see text for details; plot shows d 29 and 36 titers only.) and their sera assayed for IgG anti-HIgG antibodies.

	dose of conjugate in micrograms	number of daily injections of conjugate
A =	10	14
B =	100	"
C =	10	7
D =	100	"
E =	10	5
F =	100	"
G =	PBS	14

Days 29 and 36 of the experiment corresponds to days 14 and 21 respectively post-final immunisation.

Figure 7: EFFECT OF MULTIPLE INJECTIONS OF HIgG(mPEG)17 ON MICE RECEIVING 14 DAILY IMMUNISATIONS OF halgG

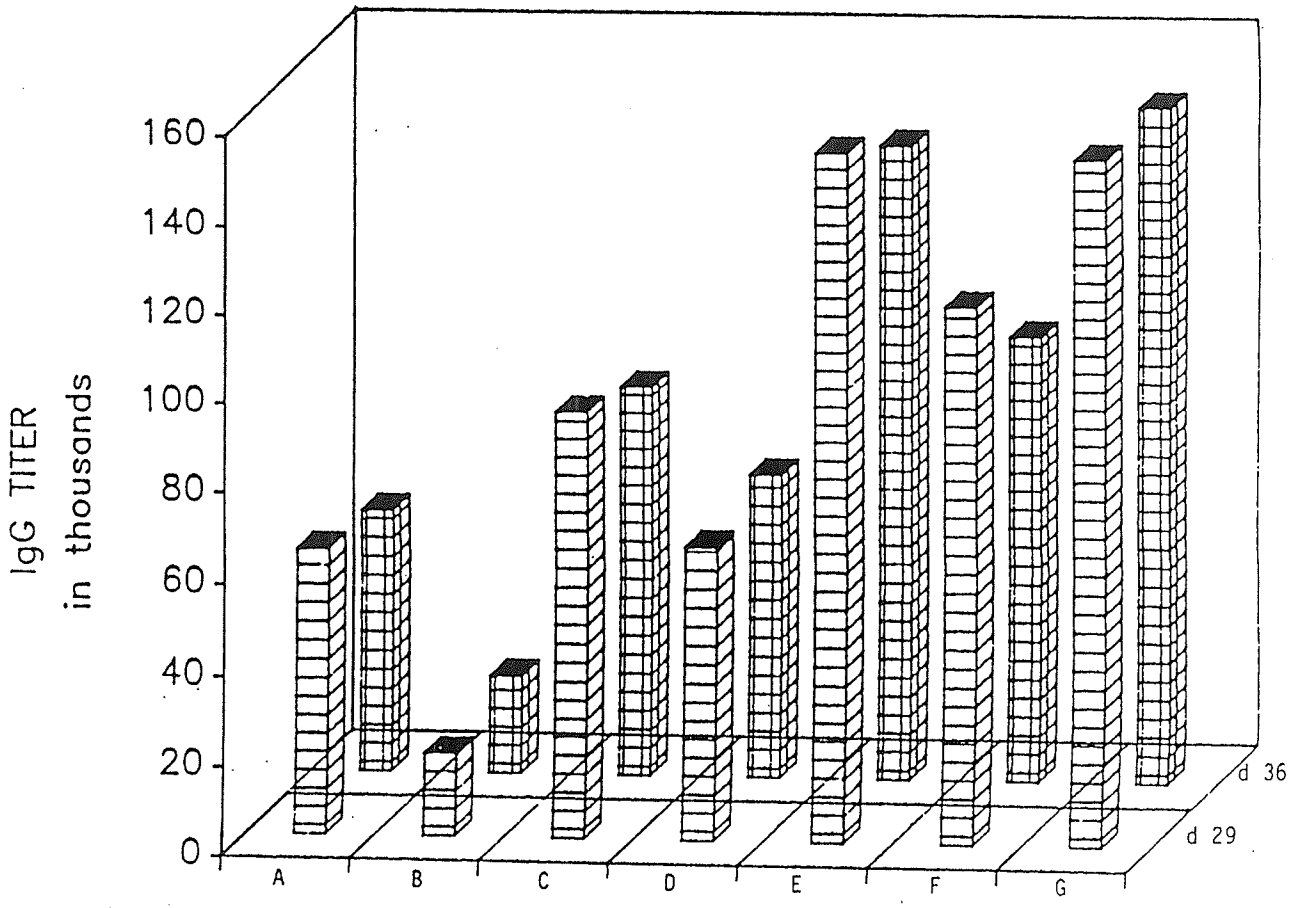


Table 8: The effect of multiple injections of HIgG(mPEG)17 on the IgM anti-HIgG response

group	number of injections post -6hr injection	Mean IgM titer (2 data points) day 15	S.D.	day 21	S.D.
b*	14	3,310	708	5,080	876
d	7	6,936	699	8,069	333
f	5	7,931	1,294	10,370	2,178
g	0	9,086	1,097	8,212	464

The experimental protocol is summarised in table 6. All mice received 14 daily injections of 50 micrograms of haIgG#2, i.p.. Groups of mice received 100 microgram doses of conjugate either daily (total = 15 injections), every second day (total = 8 injections) or every third day (total = 6 injections) for a period of 14 days. All groups receiv -ed their first injection of conjugate 6 hours prior to their first immunisation. A control group received PBS instead of conjugate. All groups were bled on the 1st, 3rd, 5th, 7th, 15th and 22nd day follow -ing their last injection of conjugate. All sera were assayed for IgM anti-HIgG#2 antibodies.

The effect of a single injection of HIgG(mPEG)17 on the IgM anti-HIgG response

group	day -6	day 0	Mean IgM titer (2 data points) day 14	S.D.	day 21	S.D.
a	conjugate	haIgG	647	132	235	57
b	PBS	"	906	161	290	94

These groups of mice were injected with 50 micrograms of HIgG(mPEG)17 or with PBS, i.v., and immunised 6 days later with 50 micrograms of haIgG#2 given i.p.. All mice were bled 14 and 21 days post-immun -isation and their sera assayed for IgM anti-HIgG#2 antibodies.

equivalent doses of either 7 or 5 daily injections.

Fourteen daily injections of haIgG#2 group (g) leads to an increased titer (181,202 for average day 15 titer) compared with a single injection (23,993) (see group (a) table 7). This titer can be reduced by multiple injections of the conjugate HIgG$(mPEG)_{17}$. The reduction in titer is related to the number of injections and to the dose of conjugate: i.e., the group that received 14 injections of 100 micrograms each of tolerogen (group (b)) showed the greatest reduction in titer (day 15 average titer of 17,069 which represents 91% suppression) and the group that received only 5 injections of 10 micrograms each of tolerogen (group(e)) showed the least reduction in titer (day 15 average titer of 152,492 which represents 16% suppression), although the titer was still less than that of the control group (g) i.e., average day 15 titer of 181,202.

Considering table 8; the IgM anti-HIgG titers of mice receiving 14 daily injections of conjugate showed suppression compared with recipients of PBS. Mice receiving either 7 or 5 injections of conjugate showed no suppression of the IgM anti-HIgG response. Mice receiving a single injection of conjugate showed no suppression compared with mice receiving PBS.

The average day 14 IgM titers for mice treated with 5, 7 or 14 injections (in addition to the injection given at -6 hr) 100 micrograms of conjugate were 7,932, 6,936 and 3,310 respectively compared with 9,086 for mice that received no conjugate but had 14 daily immunisations.

Discussion

In discussing antibody titers determined by ELISA in multiply immunised mice the effect of the high circulating levels of antigen should be considered. In experiments described previously, single injections of immunogen had been administered 14 days prior to the first bleeding day. This is between 5 and 7 half-lives for HIgG in vivo, thus negating the argument that apparently suppressed antibody titers as measured by ELISA are artifacts due to antibody being neutralised by antigen. However, in the case of multiply immunised mice (the last immunisation being only one day prior to the first bleeding) antigen neutralisation of antibody could be significant. In order to circumvent this effect all groups were bled on day 29 and 36 (i.e., 15 and 22 days after the last immunisation) thereby allowing levels of circulating antigen to fall (the half-life of the HIgG is 2.2 days therefore 15/2.2 = 6.8 half-lives or 0.8% HIgG remaining for day 29 bleedings; and 22/2.2 =10.0 half-lives or 0.01 % remaining for day 36 bleedings). Considering the data for these two days it is apparent that the suppressive effect induced by conjugate is a true phenomenon and not an artifact.

The IgG ELISA data show that the multiple injections of conjugate can reduce the response to haIgG and that the greater the number of injections the more effective the suppression. The suppression is not 100%; a titer of 17,069 remaining on the 29th day compared with a titer of around 3,000 in previously described experiments where mice received only one tolerising and one immunising injection. However,

the percent suppression is comparable: approximately 90% in both cases. In the present experiment conjugate and immunogen co-exist at higher concentrations than in experiments involving one injection of tolerogen followed six days later by one injection of immunogen. In the latter case approximately three tolerogen half-lives have elapsed before injection of immunogen. The separation in time of tolerogen and immunogen may govern the magnitude of the overall antibody response to the immunogen which may in turn reflect different mechanisms of suppression induction. In the present (multiple injection) experiment the effective concentration of tolerogen exceeds that of immunogen due, not only to different doses, but also to the decreased clearance of mPEG conjugated HIgG over non-conjugated material [See above: In vivo fate of ^{125}I-HIgG(mPEG) and ^{125}I-sham conjugated HIgG#1]. This suppression is in contrast to a single, central tolerising event that would not be effected by increasing doses of immunogen, i.e., an all or nothing situation. If this single tolerising event were the mechanism operating in experiments where one injection of tolerogen were followed 6 days later by one injection of immunogen then one might expect 100% suppression of the anti-HIgG response as opposed to 90-95% actually observed. The residual titer maybe due to anti-idiotypic antibodies that are not suppressed by the tolerogen whereas anti-isotypic and anti-allotypic antibodies are suppressed. It is also possible that the tolerogen "switches off" only lymphocytes with a high affinity for the conjugate and fails to affect low affinity cells. The latter

cells could be responsible for the residual anti-HIgG titer observed experimentally. The question of whether this residual titer would interfere with therapeutically administered antibodies remains open. However, therapeutic antibodies would not be deliberately aggregated as in the present study and would not be expected to induce an antibody response of the magnitude observed in this experimental system. Future work in this area may result in treatment protocols that produce 100 % suppression, i.e., manipulation of the number of mPEG groups per XIgG, the dose, timing and route of administration of conjugates may lead to complete (100%) tolerance induction in other strains of mice or in other species including man.

The IgM titer was also suppressed by administration of conjugate, the degree of suppression being related to the number of injections. However, the maximum suppression observed was less than that observed for IgG antibody levels, i.e., 60% compared with 91%. Mice receiving only one dose of conjugate that were also immunised only once showed 29% suppression compared with approximately 90% suppression of the IgG1 response for mice undergoing the same protocol in experiments described previously.

Hay and Torrigani (173) have shown that mice tolerised with high doses of human serum albumin show suppression of the IgG1 isotype but enhancement of the IgM isotype. In addition Shawler et al (37) have reported a progressively increasing IgM anti-MAb (in conjunction with the IgG isotype) response in patients undergoing therapy with the MAb T1010. Whilst conjugate is less effective at reducing the IgM

response than it is at suppressing the IgG response, the titers of IgM anti-HIgG antibodies are only approximately 5% of the titer for IgG anti-HIgG antibodies i.e., for day 14 the average titers are 9,086 and 181,2020 respectively (disregarding differences in specific activity of the IgM and IgG specific alkaline phosphatase coupled conjugates used in ELISA). The reason why the IgM response is not suppressed by conjugate as effetively as is the IgG response is unclear. It may be that isotypic suppression is related to the isotype of the tolerogen, i.e., IgM(mPEG)$_n$ conjugates suppress the IgM antibody response greater than the respose of other isotypes. Future studies in this area could test this hypothesis. However, such studies would require an assay system that measures each immunoglobulin isotype (and/or subclass) with equal accuracy and precision. The ELISA system used in the present study would not fullfill this requirement due to differences in specific activities of the alkaline phosphatase coupled anti-isotype antibodies employed.

Specificity of the tolerance induced by HIgG(mPEG)$_{17}$

Introduction

In order to make MAb-(mPEG)$_n$ commercially viable it would be advantageous if some limited cross tolerance were inducible, thus negating the requirement for custom conjugating a given MAb to mPEG for every MAb that is administered. Whilst in the field of allograft rejection generally applicable MAbs can be used (e.g., OKT3), this may not be the case in the wide range of tumour antigen specific MAbs

currently in use. Ruben et al (174) and Benjamin et al (141) showed that mice tolerised with high doses of deaggregated HIgG exhibited cross-tolerance when immunised with heterologous gamma globulins. In the present study the range of specificity of tolerance induction by $HIgG(mPEG)_{17}$ was examined. In part A of the experiment the effect of $HIgG(mPEG)_{17}$ upon the IgG response induced by various antigens was examined. In part B the effect of various heat aggregated antigens on the suppression induced by $HIgG(mPEG)_{17}$ was studied.

Materials and Methods

Mice given PBS or tolerised with 100 micrograms of $HIgG(mPEG)_{17}$, i.v., were immunised with 50 micrograms of the following proteins, i.p.: heat-aggregated human IgG from the human myeloma used to make conjugate $HIgG(mPEG)_{17}$ (haIgG#2), heat-aggregated human IgG from the human myeloma used to make conjugate $HIgG(mPEG)_{20}$ (haIgG#1), aggregated OA (AOA) and heat-aggregated bovine IgG (haBIgG). All groups were bled 14 and 21 days following immunisation and assayed by ELISA. The sera derived from these mice were assayed in two ways:

(A) the ELISA plates were coated with the respective non-heat aggregated antigen i.e, HIgG (10 micrograms per well) for mice immunised with haIgG, OA (20 micrograms per well) for mice immunised with AOA and BIgG (10 micrograms per well) for mice immunised with haBIgG. These assays would indicate whether or not the conjugate $HIgG(mPEG)_{17}$ could suppress the IgG response to the above mentioned immunogens.

(B) For all sera obtained from animals immunised with haIgG#1, AOA or haBIgG the plates were coated with HIgG and the effects of these antigens on HIgG specific tolerance ascertained. These assays would indicate whether or not suppression induced by $HIgG(mPEG)_{17}$ could be abrogated by any of the above mentioned immunogens.

Results

IgG anti X (where X = HIgG#2, HIgG#1, BIgG or OA) titers are presented in table 9(A) and the mean percent suppression for each group is plotted in figure 8. IgG anti-HIgG titers are presented in table 9(B).

Mice treated with 100 micrograms of $HIgG(mPEG)_{17}$ were able to suppress the anti-haIgG#1 and the anti-haIgG#2 responses and also the anti-haBIgG response, although to a lesser degree. However, mice treated with 100 micrograms of HIgG(mPEG)17 were unable to suppress the anti-AOA response.

Mice treated with 100 micrograms of conjugate and immunised with either haIgG#1, haBIgG or AOA all showed suppression with respect to the anti-HIgG response i.e.. suppression was not broken by these antigens.

In table 9(A) antibody responses to different antigens are presented, therefore, percent suppression (figure 8) must be used for purposes of comparison as opposed to actual titers. Mice tolerised with 100 micrograms of conjugate and immunised 6 days later with either haIgG#1, haIgG#2 or haBIgG all showed greater than 76%

Table 9-A: Specificity of the tolerance induced by HIgG(mPEG)17

group	d -6	d 0	IgG anti-X titer day 14	S.D.	percent suppression
a	conjugate	haIgG#2	4,060	1,322	83*
b	"	haIgG#1	3,164	545	85*
c	"	AOA	11,827	1,263	-9
d	"	haBIgG	3,711	837	76*
e	PBS	haIgG#2	24,603	1,954	-
f	"	haIgG#1	21,001	348	-
g	"	AOA	10,832	1,369	-
h	"	haBIgG	15,384	1,650	-

group	d -6	d 0	IgG anti-X titer day 21	S.D.	percent suppression
a	conjugate	haIgG#2	4,885	472	78*
b	"	haIgG#1	3,291	429	84*
c	"	AOA	12,281	1,013	-3
d	"	haBIgG	4,714	1,499	72*
e	PBS	haIgG#2	22,593	2,558	-
f	"	haIgG#1	19,977	945	-
g	"	AOA	11,968	583	-
h	"	haBIgG	17,025	602	-

Table 9-B: Specificity of the tolerance induced by HIgG(mPEG)17

group	d -6	d 0	IgG anti-HIgG titer day 14 mean	S.D.	percent suppression
b	conjugate	haIgG#1	4,574	1,636	81*
c		AOA	5,024	1,401	80*
d	"	haBIgG	5,076	1,223	79*

group	d -6	d 0	IgG anti-HIgG titer day 21 mean	S.D.	percent suppression
b	conjugate	haIgG#1	4,621	861	80*
c		AOA	6,569	221	71*
d	"	haBIgG	5,091	1,119	77*

Percent suppression = 100 - (titer for HIgG(mPEG)17 treated mice on d -6 / titer for PBS treated mice on day -6) x 100. Mice were injected with 100 micrograms of HIgG(mPEG)17 or PBS, i.v. and then immunised 6 days later with one of the following proteins (i.p.): haIgG#1 (10 micrograms) or haIgG#2 (50 micrograms) or AOA (50 micrograms) or haBIg

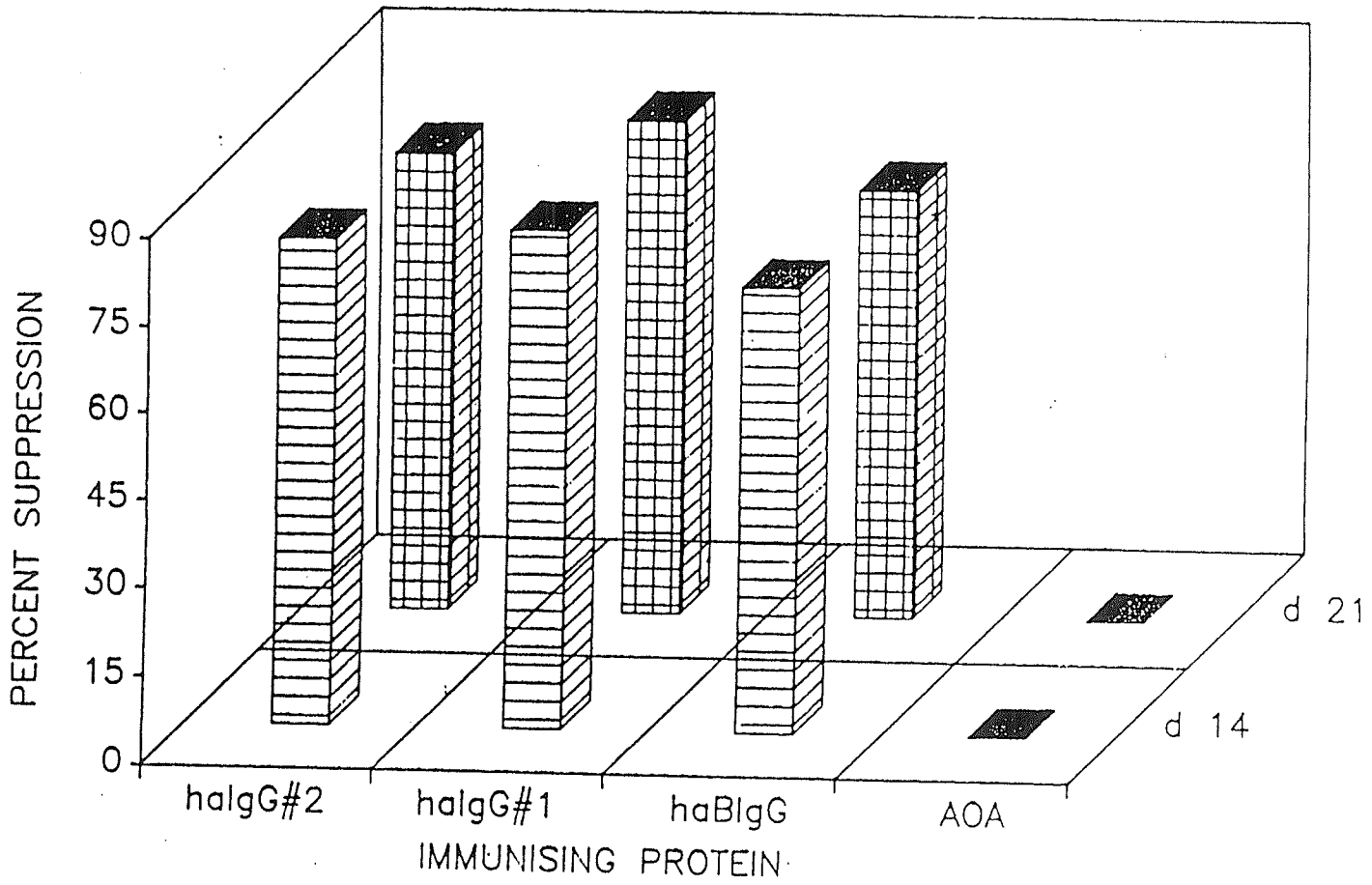
50 micrograms). All groups were bled 14 and 21 days post-immunisation All sera were assayed in two different ways:
(a) Sera were assayed on ELISA plates coated with their corresponding protein, i.e., HIgG for mice immunised with haIgG; OA for mice immunis -ed with AOA; and BIgG for mice immunised with haBIgG. (see table 9-A)
(b) Sera were assayed on ELISA plates coated with HIgG irrespective of the immunising protein.

Figure 8: Legend
Specificity of the tolerance induced by HIgG(mPEG)$_{17}$

Groups of mice were injected with either 100 micrograms of HIgG(mPEG)$_{17}$ or PBS, i.v. and then immunised 6 days later with one of the following proteins (i.p.): haIgG#1 (10 micrograms) or haIgG#2 (50 micrograms) or AOA (50 micrograms) or haBIgG (50 micrograms). All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-X antibodies (where X = HIgG#1, HIgG#2, BIgG or OA).

Percent suppression = 100 - (titer for group treated with conjugate on day -6/ titer for group treated with PBS on day -6) x 100.

Figure 8: SPECIFICITY OF THE TOLERANCE INDUCED BY HIgG(mPEG)17



suppression of their respective responses whereas mice immunised with AOA showed no suppression of the IgG anti-AOA response. All percentage values are with respect to control groups that received PBS in place of conjugate and were immunised 6 days later with the one of the above mentioned heat-aggregated proteins.

Considering table 9(B) i.e., anti-HIgG titers, it is apparent that immunisation with haIgG#1, haBIgG or with AOA fails to terminate the suppression induced by HIgG(mPEG)$_{17}$

Discussion

The cross-tolerance to heterologous immunoglobulins observed in mice receiving 100 micrograms of conjugate implies that it may be unnecessary to synthesise MAb-(mPEG)$_n$ conjugates for every MAb in clinical use. These observations together with the lack of cross-tolerance in the groups receiving aggregated OA suggests that there is some similarity between determinants (immunodominant determinant(s)) on the different immunoglobulins.

Benjamin (141) has demonstrated cross-tolerance using mice tolerised with high doses of deaggregated HIgG and immunised with heterologous immunoglobulins; and that this cross-reactivity also extends to the T suppressor cells generated by the administration of deaggregated HIgG. He postulated that the determinants recognised on HGG and BGG by HGG induced suppressor cells may be essentially identical. Thus, it is possible that HIgG(mPEG)$_{17}$ induces cross-reacting suppressor cells that suppress the immune response to

closely related molecules, i.e., HIgG#1 and BIgG but not to unrelated molecules, i.e., OA.

Chapter III

Examination of mechanism(s) of tolerance induction by XIgG(mPEG)$_n$ conjugates

Introduction

In the previous section it was demonstrated that 100 micrograms of the conjugate HIgG(mPEG)$_{20}$ administered i.v., gave the greatest amount of suppression for the conditions stated. This conjugate was therefore selected for further studies aimed at elucidating the mechanism(s) of tolerance induction by HIgG(mPEG)$_n$ conjugates. In order to examine whether or not the observed phenomena were peculiar to the the myeloma HIgG#1 the conjugate HIgG(mPEG)$_{17}$ was also utilised.

Transfer of tolerance by splenic leukocytes from tolerised mice

Introduction

Suppressor cells are able to suppress the function of B cells or other T cells. They may function by inactivating either T or B cell clones possibly via soluble factors. The action of suppressor cells may be demonstrated by transferring tolerance to a recipient animal by suppressor cells ("infectious tolerance"). Since HGG specific suppressor cells have been demonstrated by Basten et al (129) and by Benjamin et al (130) it was hypothesised that HIgG(mPEG)$_n$ conjugates may also induce suppressor cells.

The transfer of splenic leukocytes, subpopulations of splenic leukocytes and freeze/thaw extracts of these cells, from tolerant

into naive (non-irradiated) recipients allows the expression of their suppressive abilities to be studied in a situation similar to that in the intact tolerant animal. Weigle (175) has suggested that irradiation has the effect of enhancing the termination of unresponsiveness, possibly due to the pressure to proliferate among the surviving cells (either those of the irradiated recipient or those from the donor animal). Indeed, Jones and Kaplan (124) reported no difficulties in the transfer of tolerance by spleen cells derived from HGG tolerant donors into naive syngeneic recipients whereas Basten et al (129) and Doyle et al (137) reported difficulty in transferring tolerance using adoptive transfer into irradiated recipients.

Materials and Methods

Mice were tolerised with 100 micrograms of HIgG$(mPEG)_{20}$ given i.v. and sacrificed 6 days later. Their spleens were removed, trimmed of excess fat and placed in Hepes-MEM pH = 7.2. A cell suspension was produced by gentle rotation of a teflon pestle against the side of the tube holding the cells. The suspension was filtered through a coarse mesh stainless steel screen and then through a fine mesh screen. After washing three times in MEM, 20 mls of the suspension containing the cells from 3 spleens were layered onto 14 ml of Ficol-Hypaque (density = 1.092 g/ml) and centrifuged at 1,600 rpm for 15 minutes at RT. The layer of cells at the Ficol/MEM interface was removed with a pasteur pipette and washed three times before

being counted in a hemocytometer and their viability checked by the trypan blue exclusion method. Each recipient mouse received 5 x 10^7 splenic leukocytes in 0.5 ml of MEM. Control groups received the same number of splenic leukocytes derived from normal mice (normal leukocytes) i.e., donor mice that had been given PBS in place of conjugate. Further recipient control groups were not given any cells but received instead either MEM or PBS (0.5 mls), i.v.. All recipients groups were immunised one day later and bled 14 and 21 days post-immunisation.

Results

The IgG1 titers are presented in table 10 and the means are plotted in figure 9. [Please note that table 10 and figure 9 also contain data from experiments described below. These data are presented together in order to facilitate comparison.] Mice receiving either PBS or MEM showed no suppression with average day 14 titers of 19,193 and 18,125 respectively. Recipients of normal leukocytes showed no suppression on average. Mice receiving tolerised leukocytes showed significant suppression with an average day 14 titer of 3,135. This transferable suppression was shown to be specific because recipients of tolerised leukocytes were unable to suppress the anti OA response when these mice were immunised with AOA.

Discussion

HGG specific suppressor cells were first described in tolerant

Table 10: Transfer of tolerance by leukocytes from donor mice toleris -ed with 100 micrograms of HIgG(mPEG)20

History day -6	donor day 0	recipient day 1	Mean IgG1 anti-HIgG titer (groups g & h = anti-OA titer) day 14	S.D.	day 21	S.D.
(a) -	PBS	haIgG	19,193	2,739	19,763	3,298
(b) -	MEM	"	18,125	2,538	19,680	2,986
(c) PBS	cells (1)	"	18,096	3,078	18,387	4,732
(d) conj.	cells (2)	"	3,135	515	3,296	957
(e) "	cells (3)	"	2,011	172	2,595	1,218
(f) "	cells (4)	"	13,859	3,883	15,264	3,898
(g) "	cells (5)	AOA	7,527	4,342	8,471	5,268
(h) PBS	Cells (6)	"	8,197	4,902	7,310	4,296

(1) = 5 x 10^7 normal splenic leukocytes from naive BDF1 mice donors
(2) = 5 x 10^7 splenic leukocytes from mice tolerised with 100 micro -grams of HIgG(mPEG)20 six days before transfer into recipient.
(3) = 2 x 10^7 sIg- splenic leukocytes obtained as in (2) above but with additional fractionation of leukocytes into sIg- and sIg+ populations using petri dishes coated with sheep anti-mouse Ig.
(4) = 3 x 10^7 sIg+ splenic leukocytes obtained as in (3) above.
(5) = 5 X 10^7 splenic leukocytes obtained as in (2) above but note the recipients of these cells were immunised with AOA instead of haIgG as in (2).
(6) = 5 x 10^7 normal cells obtained as in (1) above but note that the recipients of these cells were immunised with AOA instead of haIgG as in (1).
Conjugate was administered i.v. (100 micrograms) and immunisations were administered i.p. (10 micrograms of haIgG or 50 micrograms of AOA).

Group	number of data points
(a)	9
(b)	14
(c)	8
(d)	12
(e)	6
(f)	4
(g)	4
(h)	4

Figure 9: Legend
Transfer of tolerance by cells from donors treated with $HIgG(mPEG)_{20}$

Groups of mice were injected, i.v., with either $HIgG(mEG)_{20}$ or PBS and killed 6 days later. Various populations (fractions) of splenic leukocytes were transferred, i.v., into naive syngeneic recipients as follows:

PBS = recipients were injected with PBS only

MEM = recipients were injected with MEM only

Normal = recipients were injected with splenic leukocytes derived from donor mice injected with PBS only. Transferred 5×10^7 cells.

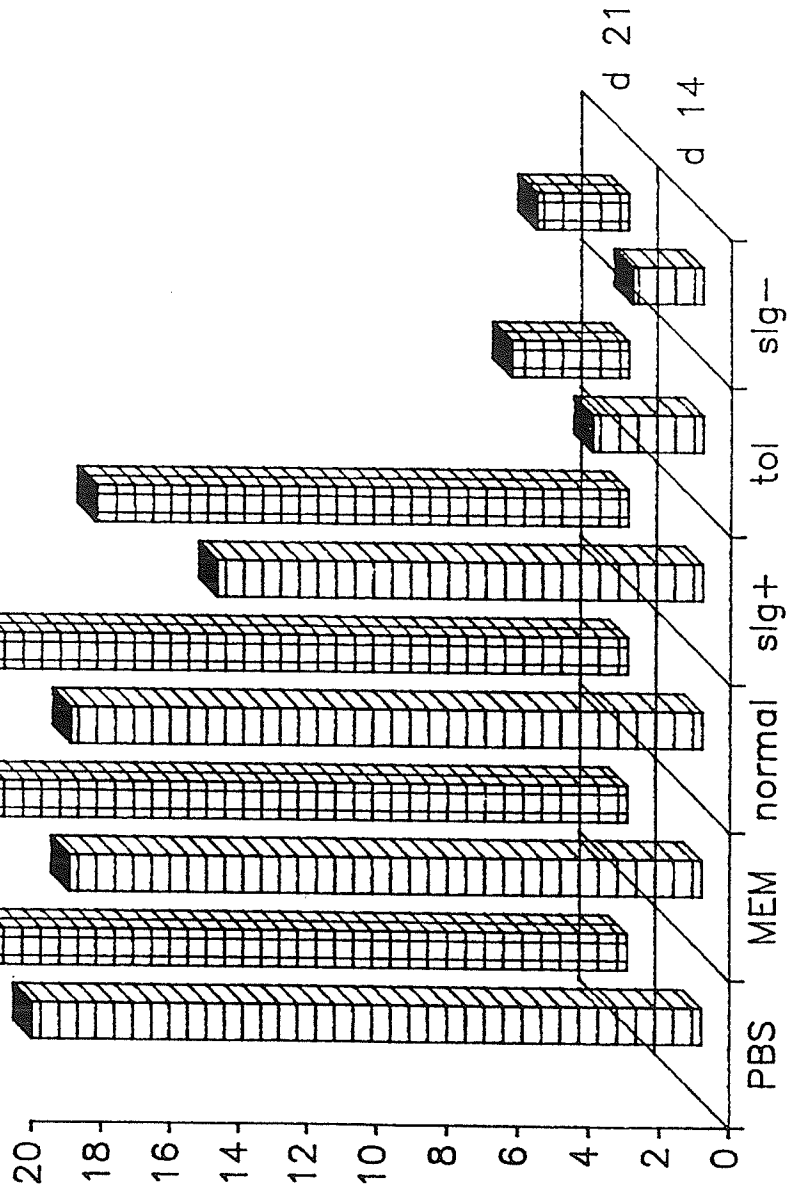
sIg+ = recipients were injected with splenic leukocytes derived from donors injected with conjugate and fractionated by incubation on petri dishes coated with sheep anti-mouse Ig. Transferred 2×10^7 cells.

tol = recipients were injected with splenic leukocytes derived from donor mice injected with conjugate. Transferred 5×10^7 cells.

sIg- = recipients were injected with the fraction of cells that did not adhere to the petri dishes above. Transferred 2×10^7 cells.

Groups were immunised with 10 micrograms of haIgG#1 or AOA one day after receiving cells. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Figure 9: TRANSFER OF TOLERANCE BY CELLS FROM DONORS TREATED WITH HIgG(mPEG)20



mice by Basten et al (129), Benjamin (130) and Doyle et al (131,137). Basten investigated the effects of HGG specific suppressor cells upon HGG primed helper T cells and DNP primed B cells by immunising reconstituted recipients with DNP-HGG. Benjamin (130) and Doyle et al (131,137) examined the suppression of both HGG specific helper T cells and B cells using a similar system. These HGG specific suppressor cells were shown to be capable of suppressing both HGG specific T and B cells in vivo. HGG primed mice also have detectable antigen specific suppressor cells (Loblay et al (138)). This suggests that these suppressor cells are a part of a normal regulatory mechanism activated by antigenic challenge;

The data presented here together with the antigen carry-over experiment described below demonstrate the presence of HIgG specific suppressor cells in mice tolerised with $HIgG(mPEG)_{20}$.

Estimation of amount of tolerogen carried-over in transfer experiments

Introduction

The possibility that transfer of tolerance was due to carry-over of tolerogen (either per se or in altered form) rather than by cells was investigated. A radioiodinated (^{125}I) derivative of $HIgG(mPEG)_{20}$ was synthesised by the chloramine-T method (168) as previously described and used to tolerise donor animals. Cells or F/T extracts of cells were obtained from these mice 6 days later and aliquots transferred into naive syngeneic recipients.

Materials and Methods

HIgG$(mPEG)_{20}$ was radioiodinated with ^{125}I using the chloramine-T method (see Material and Methods-General section) and unbound iodine removed by passage through a G-25 gel filtration column. The radiolabelled material was diluted with cold conjugate such that there were 0.73×10^7 cpm per 100 micrograms of conjugate. This was given i.v. into donor mice which were killed 6 days later; and splenic leukocytes, sIg- and sIg+ leukocytes and F/T extract of sIg- leukocytes obtained as described below. The cpm associated with each cell type or extract was determined using a Beckman 300 Gamma counter and background counts subtracted.

In order to determine if these radiolabelled cells and extracts were immunologically equivalent to `cold' cells and extracts in vivo, aliquots of each cell type, extract or MEM were injected i.v., into naive syngeneic recipients. Three groups received 1, 0.1 or 0.01 micrograms respectively of `cold' tolerogen. All groups were immunised one day later and bled 14 and 21 days post-immunisation.

Results

The radioactivity data obtained are summarised in table 11. Recipients of tolerised splenic leukocytes received 4.22 or 9.97 ng of conjugate compared with 0.59 or 0.85 ng in those receiving sIg- tolerised splenic leukocytes and 0.34 or 0.57 ng in those receiving F/T extract derived from the latter cells.

The in vivo data are summarised in table 12. Recipients of

Table 11:
Estimation of amount of tolerogen carried over in the transfer of tolerance by cells and freeze/thaw (F/T) extracts derived from donor mice tolerised with HIgG(mPEG)20

	Mean values (2 data points per mean)			
cell or extract	# of cells received ($\times 10^8$)	cpm	conjugate (ng)	conjugate per recip -ient (ng)
(a) leukocytes	38.55	3,638	53.42	7.10
(b) sIg-	5.40	133	1.96	0.72
(c) sIg+	12.70	429	6.30	1.49
(d) sIg-/rHIgG-	0.75	84	1.24	0.33
(e) sIg-/rHIgG+	0.90	13	0.20	0.05
(f) F/T (sIg-)	2.00	31	0.46	0.46
(g) F/T(sIg-/rHIgG+)	0.20	5	0.08	0.08

Each mouse received 7.3×10^7 cpm / 100 ug ofHIgG(mPEG)20 corrected for decay of the isotope per se . Thus, after 6 days = $0.93 \times 7.3 \times 10^7 = 6.8 \times 10^7$.
All cpm are minus background.

Donor mice were tolerised with 100 microgam doses of HIgG(mPEG)20 i.v that had been radiolabelled. These mice were killed and their splenic leukocytes transferred into syngeneic naive recipients or further fractionated into the following cell populations:

(a) = 5×10^7 splenic leukocytes from donor mice tolerised with 100 micrograms (i.v.) of radiolabelled HIgG(mPEG)20.
(b) = 2×10^7 sIg- splenic leukocytes fractionated from the cell population in (a)above using petri dishes coated with sheep anti-mouse Ig.
(c) = 2×10^7 sIg+ splenic leukocytes obtained as in (b) above.
(d) = 2×10^7 sIg- splenic leukocytes obtained as in (b) above but then further fractionated into rHIgG+ population using petri dishes coated with HIgG.
(e) Freeze/thaw extract derived from 2×10^7 sIg- cells obtained as in (b) above.
(f) Freeze/thaw extract derived from 2×10^7 sIg-/rHIgG+ cells obtain -ed as in (d) above.

Table:12
In vivo testing of the tolerogenic capacity of cells and freeze/thaw (F/T) extracts obtained from donor mice given radiolabelled conjugate

group	Mean IgG1 anti-HIgG titer (2 data points per mean) day 14	S.D.	day 21	S.D.
(a)* leukocytes	3,265	565	3,376	864
(b)* sIg-	2,083	338	3,441	1,227
(c)* sIg+	16,483	2,091	18,368	1,521
(d)* sIg-/rHIgG-	14,199	487	13,015	1,390
(e)* sIg-/ rHIgG+	2,627	586	2,321	486
(f)* F/T (sIg-)	2,427	651	3,611	1,476
(g)* F/T (sIg-/HIgG+)	2,533	95	2,819	387
(h) MEM	21,164	1,794	21,488	1,335

(a) = 5×10^7 splenic leukocytes from donor mice tolerised with 100 micrograms (i.v.) of radiolabelled HIgG(mPEG)20.
(b) = 2×10^7 sIg- splenic leukocytes fractionated from the cell population in (a)above using petri dishes coated with sheep anti-mouse Ig.
(c) = 2×10^7 sIg+ splenic leukocytes obtained as in (b) above.
(d) = 2×10^7 sIg- splenic leukocytes obtained as in (b) above but then further fractionated into rHIgG- population using petri dishes coated with HIgG.
(e) = 2×10^7 sIg-/rHIgG+ splenic leukocytes obtained as in (d) above
(f) Freeze/thaw extract derived from 2×10^7 sIg- cells obtained as in (b) above.
(g) Freeze/thaw extract derived from 2×10^7 sIg-/rHIgG+ cells obtain -ed as in (d) above.
(h) MEM = control. MEM was used throughout as a diluent for all cell populations and freeze/thaw extracts.

Table 12-B:
Effect of low doses of HIgG(mPEG)20 on the induction of tolerance

Dose	conjugate	Mean IgG1 anti-HIgG titer (2 data points per mean) day 14	S.D.	day 21	S.D.
1 µg	HIgG(mPEG)20	16,402	2,162	16,323	114
0.1 µg	"	19,410	1,546	20,131	1,532
0.01 µg	"	18,621	2,281	20,807	999

Conjugate was given i.v. and mice were immunised 6 days later with 10 micrograms of halgG, i.p.

leukocytes, sIg-, sIg-/rHIgG+ and F/T_{20} all showed suppression compared with control groups that received only MEM. Recipients of sIg+ cells from donor mice treated with conjugate and recipients of less than 1 micrograms of tolerogen showed no suppression. There was no correlation between the amount of radiolabelled conjugate transferred and the degree of suppression observed in the recipients.

Discussion

It is possible that the radiolablel was excised from the rest of the tolerogen (or derivative thereof) in vivo and that there is no correlation between cpm and amount of conjugate present. In addition it is possible that the radioiodination of conjugate molecules may not occur with equal probability, i.e., in a conjugate designated as $HIgG(mPEG)_n$ there will in reality be a distribution of degrees of substitution about N. If this is so, then the radiolabelled conjugate molecules may not be representative of the whole population. These limitations of the method must be born in mind when interpreting data derived from experiments of this type.

Mice receiving < 1 micrograms of tolerogen showed no suppression. These observations in conjunction with the data presented in table 10, indicate that the minimum amount of conjugate required to directly tolerise a mouse is 10 - 100 micrograms. However, mice receiving leukocytes apparently bearing the equivalent of 9.97 ng (approximately 0.01 micrograms) of conjugate showed suppression. As these recipient mice received doses of tolerogen approximately 100

fold lower than the amount of conjugate required to directly induce tolerance in intact animals it appears that transfer of tolerance is not due to tolerogen carry-over. [see also chapter IV, Tolerogen carry-over during transfer of tolerance by F/T_{20}, for a discussion of the interpretation of data obtained using iodinated materials].

Relationship between duration of tolerance in intact mice and transfer of tolerance by splenic leukocytes

Introduction

In order to determine the relevance of the presence of suppressor cells in $HIgG(mPEG)_{20}$ tolerant mice, the duration of transferable tolerance was compared with the duration of tolerance in `intact' mice.

Materials and Methods

Mice were tolerised and splenic leukocytes obtained as described previously. In the `intact' groups of mice the interval between the administration of conjugate and immunisation was 15, 29, 43 or 57 days. In the transferred groups of mice 5×10^7 splenic leukocytes from tolerised donor mice were transferred into naive syngeneic recipients either 6, 14, 28 or 42 days following administration of conjugate. Recipient mice were all immunised one day after injection of the cells; thus both the `intact' groups of mice and the `transfer' groups of mice were immunised on the same days. Control groups received PBS in place of conjugate or spleen cells. All groups were bled 14 and 21 days following immunisation.

Results

The IgG1 titers are presented in table 13 and the means for immunisation days 15, 29, 43 and 57 are plotted in figure 10. Tolerance induced by 100 micrograms of HIgG$(mPEG)_{20}$ persists for 43 days (80% suppression) after administration of conjugate; and some suppression is still observable for up to 57 days (49% suppression). However, transferable tolerance can be demonstrated for only 15 days (72% suppression) following administration of conjugate. Calculations of percent suppression take the mean titers for groups receiving PBS in place of conjugate as 100% response.

Discussion

It is apparent from the above data that there is a disparity between the duration of transferable suppression and the duration of tolerance in intact mice. This is in agreement with the findings of Basten et al (129), Doyle et al (137) and Loblay et al (138) who have all shown that HGG specific suppressor cells can be detected for a short period (approximately 40 days) relative to the duration of unresponsiveness in T (approximately 130 days) and B cells (approximately 45 days). Parks and Weigle (127) infer from these findings that suppressor cells have little if any responsibility for the maintenance and termination of unresponsiveness in either splenic T or B cells. However, the demonstration of memory suppressor cells by Loblay et al (142) raises the possibility that once the initial suppressor cells have waned in tolerised animals, memory suppressor

Table 13:
The duration of transferable tolerance induced by HIgG(mPEG)20

	# of days post tolerisation		Mean IgG1 anti-HIgG titer (2 data points per mean)			
Donor	transfer	haIgG	day 14	S.D.	day 21	S.D.
(a)* Tol	14	15	4,829	1,591	3,755	785
(b) Tol	28	29	13,166	1,760	14,545	1,366
(c) Tol	42	43	15,140	1,923	14,937	4,105
(d) PBS	-	6	17,874	1,486	19,696	677

Donor mice were tolerised with 100 micrograms of HIgG(mPEG)20 i.v. and then killed 14 days (a), 28 days (b) or 42 days (c) later 5×10^7 splenic leukocytes were then transferred, i.v., into recipient Recipients were immunised one day later with 10 micrograms of haIgG i.p.. Recipients were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.
A control group (d) received PBS in place of conjugate. 5×10^7 splenic leukocytes were transferred 6 days later and recipients were immunised as above.

The duration of tolerance induced by HIgG(mPEG)20 in intact mice

	# of days post tolerisation	Mean IgG1 anti-HIgG titer (2 points per mean)			
Donor	haIgG	day 14	S.D.	day 21	S.D.
(e)* Tol	15	2,804	448	3,163	355
(f)* Tol	29	4,066	1,486	4,285	729
(g)* Tol	43	3,310	1,582	4,466	1,069
(h) Tol	57	8,326	839	7,351	806
(i) PBS	6	16,365	1,677	17,620	1,867

Mice were tolerised with 100 micrograms ofHIgG(mPEG)20 or given PBS i.v. and then immunised with 10 micrograms of haIgG, i.p., 15 days (group (e)), 29 days (group (f)), 43 days (group (g)) or 57 days (group (h)) later. A control group (i) received PBS in place of con -jugate and was immunised as above, 6 days later. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Figure 10: Legend
The relationship between duration of tolerance in intact mice and transfer of tolerance by splenic leukocytes.

Transfer
Donor mice were tolerised with 100 micrograms of $HIgG(mPEG)_{20}$, i.v. and then killed 14, 28 or 42 days later. $5^{20} \times 10^{7}$ splenic leukocytes were transferred, i.v., into naive syngeneic recipients which were then immunised with 10 micrograms of haIgG#1 the following day (thus immunisation day = transfer day+1). All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies. A donor mice control group received PBS in place of conjugate.

Intact
Donor mice were tolerised with 100 micrograms of $HIgG(mPEG)_{20}$, i.v. and then immunised with 10 micrograms of haIgG#1, i.p., 15, 29 43 or 57 days later. Acontrol group received PBS in place of conjugate and was immunised as above six days later. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

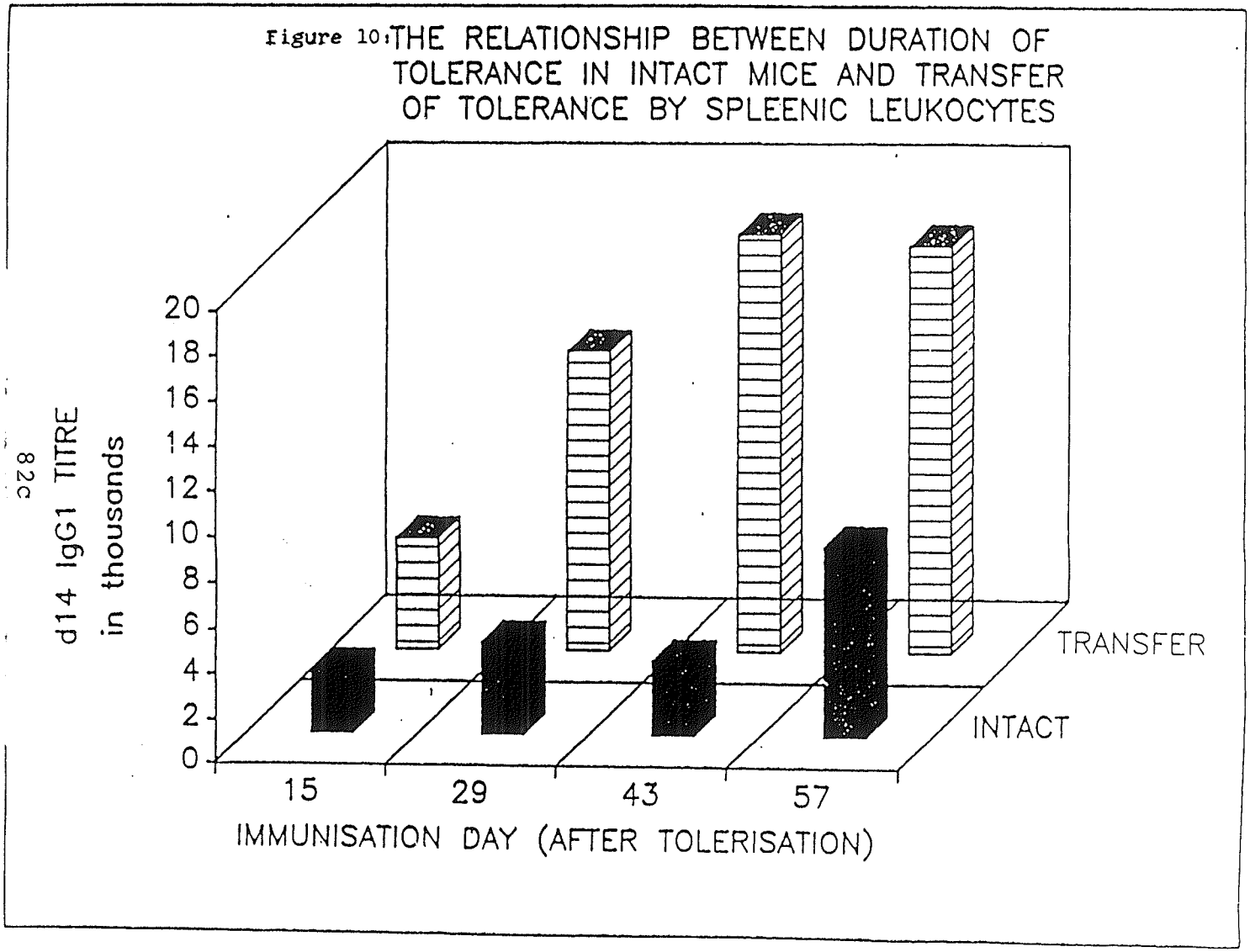


Figure 10: THE RELATIONSHIP BETWEEN DURATION OF TOLERANCE IN INTACT MICE AND TRANSFER OF TOLERANCE BY SPLEENIC LEUKOCYTES
d14 IgG1 TITRE in thousands
0
2
4
6
8
10
12
14
16
18
20
15
29
43
57
IMMUNISATION DAY (AFTER TOLERISATION)
TRANSFER
INTACT

cells may remain which are only detectable when the animal again encounters antigen. Suppressor cell memory is discussed further below.

Absence of evidence for suppression memory in mice tolerised with $HIgG(mPEG)_{20}$

Introduction

The possibility that suppressor cells were indeed relevant to the maintenance of tolerance in the $HIgG(mPEG)_{20}$ system, but were present as memory suppressor cells in the period following the transient production of suppressor cells, was investigated. The following experiments were carried out in an attempt to demonstrate specific suppression memory in mice treated with $HIgG(mPEG)_{20}$. If memory suppressor cells were to be generated by administration of two widely separated injections of conjugate then it would be expected that the suppression induced by transfer of splenic leukocytes would (a) appear more rapidly; (b) be more profound and (c) be of longer duration in comparison with animals that had received only one injection of the conjugate.

Materials and Methods

Mice were given two doses of 100 micrograms each of $HIgG(mPEG)_{20}$, 57 days apart (i.e., an interval shown previously to be long enough for the tolerance induced by the first injection of conjugate to be waning. Control groups received PBS at the first injection and the conjugate at the second or PBS on both occasions. These mice were

sacrificed and 5×10^7 splenic leukocytes transferred into naive syngeneic recipients either 2, 4 or 6 days following the second administration of conjugate. Recipients were immunised the following day and bled 14 and 21 days post-immunisation.

In a parallel experiment mice were given two injections of conjugate 57 days apart and immunised 6 days after the second administration of conjugate. These groups were bled 4, 10, 14 and 21 days post-immunisation. control groups received PBS in place of conjugate at the first injection.

Results

The IgG1 titers for the transfer experiment are presented in table 14 (upper section) and the means are plotted in figure 11. The IgG1 titers for the parallel experiment on intact mice are presented in the lower section of table 14, and the means are plotted in figure 12.

Mice receiving two 100 micrograms injections of $HIgG(mPEG)_{20}$ spaced 57 days apart (the first being 63 days prior to immunisation) showed no suppression compared with mice receiving PBS. Mice receiving a single injection of conjugate 63 days prior to immunisation also showed no suppression. Mice given a single injection of conjugate 6 days prior to immunisation showed suppression.

Recipients of spleen cells derived from donor mice given two 100 micrograms injections of $HIgG(mPEG)_{20}$ spaced 57 days apart, showed

Table 14-A:Absence of evidence for suppression memory in intact mice

				Mean IgG1 anti-HIgG titer (2 data points per mean)				
d0	d57	d63	d67	d73	d 77	S.D.	d 84	S.D.
tol	tol	haIgG	<320	12,950	21,668	3,298	22,301	5,658
PBS	tol	"	"	480	2,626	2,246	3,056	102
tol	PBS	"	"	9,505	21,302	799	22,731	4,534
PBS	PBS	"	"	13,325	21,301	1,980	22,901	4,243

Mice received either 100 micrograms of HIgG(mPEG)20, i.v., on days 0 and day 57 or on only one of those days. A control group received only PBS on both occasions. All groups were immunised on day 63 as indicated with 10 micrograms of haIgG, i.p. and then bled 14 and 21 days post-immunisation. All sera were assayed for IgG1 anti-hIgG antibodies. Note: titers for d 67 were <320, i.e., less than the lowest dilution of the ELISA.

Table 14(B):
THE EFFECT OF A SECOND INJECTION OF TOLEROGEN ON THE TRANSFER OF TOLERANCE

				Mean IgG1 anti-HIgG titer (2 data points per mean)			
d 0	d57	Transfer day	Immunise day	day 74	S.D.	day 81	S.D.
TOL	TOL	59	60	21,790	4,651	24,065	1,329
PBS	TOL	59	60	8,775	1,411	10,039	1,157
TOL	TOL	61	62	8,402	1,816	9,667	3,147
PBS	TOL	61	62	2,791	1,124	3,410	933
TOL	TOL	63	64	4,913	827	3,357	646
PBS	TOL	63	64	974	642	2,391	875
PBS	PBS	61	62	18,139	2,968	20,270	3,902

Mice received either 100 micrograms of HIgG(mPEG)20, i.v., on days 0 and day 57 or on only one of those days. A control group received only PBS on both occasions. Donor mice were killed 2, 4 or 6 days following their day 57 injection and 5 x 10^7 of their splenic leuko-cytes transferred, i.v. into recipients, which were then immunised as indicated with 10 micrograms of haIgG, i.p. and then bled 14 and 21 days post-immunisation. All sera were assayed for IgG1 anti-hIgG antibodies.

Figure 11: Legend
Absence of evidence for memory suppressor cells in mice treated with $HIgG(mPEG)_{20}$

Mice received either 100 micrograms of $HIgG(mPEG)_{20}$, i.v., on days 0 and 57 or on only one of those days. If they did not receive conjugate, PBS was injected instead. A control group received PBS on both occasions. Donor mice were killed 2, 4, or 6 days following their day 57 injection and 5×10^7 of their splenic leukocytes transferred, i.v., into recipients. Recipients were immunised the following day with 10 micrograms of haIgG#1, i.p., bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

P = PBS
T = tolerogen, i.e., $HIgG(mPEG)_{20}$

P,P = PBS on day 0 and PBS on day 57
T,T = tolerogen on day 0 and on day 57
P,T = PBS on day 0 and tolerogen on day 57

Figure 11 ABSENCE OF EVIDENCE FOR MEMORY SUPPRESSOR CELLS IN MICE TREATED WITH HIgG(mPEG)20

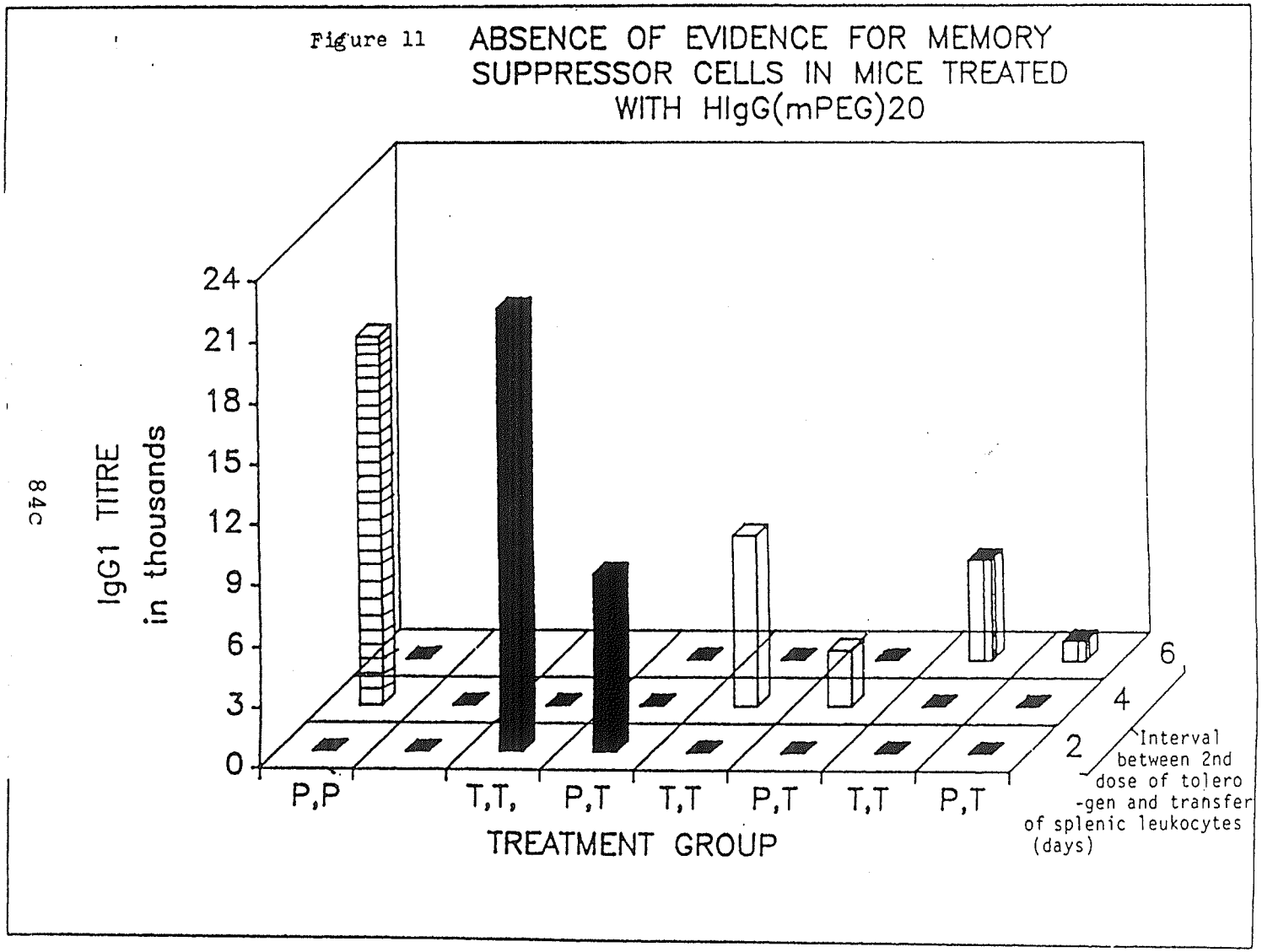


Figure 12: Legend

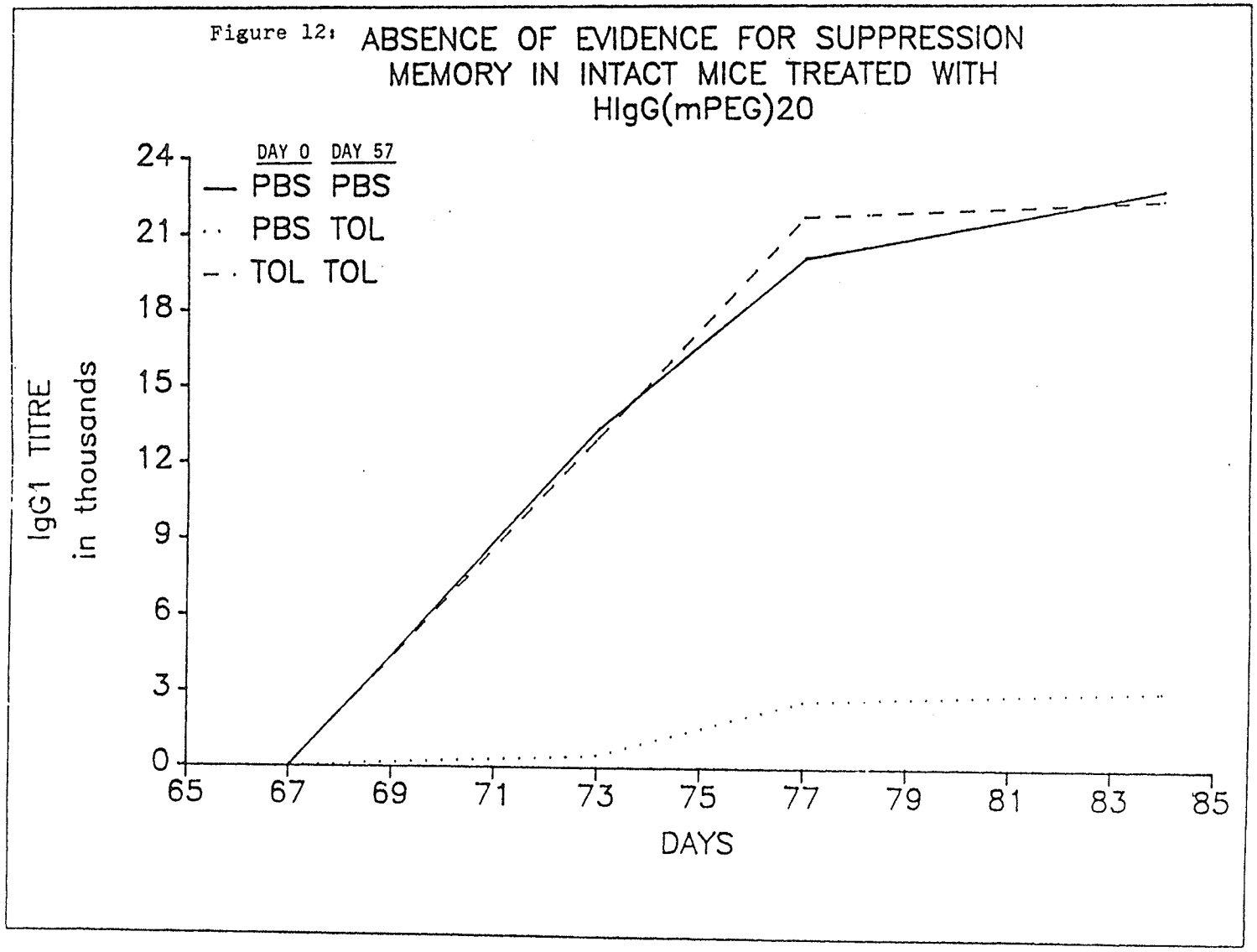
Absence of evidence for suppression memory in intact mice treated with $HIgG(mPEG)_{20}$

Mice received either 100 micrograms of $HIgG(mPEG)_{20}$, i.v., on days 0 and 57 or on only one of those days. If they did not receive conjugate, PBS was injected instead. A control group received PBS on both occasions. All groups were immunised on day 63 with 10 micrograms of haIgG#1, i.p., bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Tol = tolerogen, i.e., $HIgG(mPEG)_{20}$

PBS PBS = PBS on day 0 and PBS on day 57
TOL TOL = tolerogen on day 0 and on day 57
PBS TOL = PBS on day 0 and tolerogen on day 57

Figure 12: ABSENCE OF EVIDENCE FOR SUPPRESSION MEMORY IN INTACT MICE TREATED WITH HIgG(mPEG)20



no suppression compared with mice receiving PBS, if leukocytes were transferred 2 days following the second injection of conjugate; however similarly treated mice which received leukocytes either 4 or 6 days following the second injection of conjugate did show suppression. This suppression was similar to that observed for equivalent groups of mice treated only once with conjugate.

Considering the transfer data, it is apparent that the recipients of cells from donors treated with tolerogen twice were not suppressed to the same degree as those from donor mice given only one injection of conjugate (21,790 compared with 8,775, 8,402 compared with 2,791, et.c). In addition, no accelerated appearance of suppressor cells was observed in the doubly treated group i.e., 4,913 compared with 974 for the singly treated group for 6 days following injection of tolerogen. If memory suppressor cells were present it would be expected that suppression would be transferable less than six days following the second injection of conjugate.

Considering the parallel experiment in intact mice, it is apparent that tolerance persists for longer in the group that received only one injection of conjugate on day 57. A titer of 21,668 was measured 20 days following the second administration of conjugate compared with a titer of 2,626 for the group receiving only one injection of conjugate.

Discussion

Loblay et al (142), using high doses (3 mg) of deaggregated

HGG, have demonstrated the existence of suppressor cells in two distinct functional states: effector cells and memory cells. The former are those transiently produced during tolerance induction. Suppressor memory cells on the other hand do not express effector functions in transfer experiments unless they are first reactivated by secondary antigenic stimulation. The data presented here show no evidence for suppressor memory cells under this set of conditions. Indeed, administration of two widely separated doses of conjugate appears to reduce the degree of suppression compared with administration of only one dose. The observation that splenic leukocytes derived from mice injected with a single dose of tolerogen 6 days before sacrifice are capable of transferring tolerance, refutes the argument that T suppressor cells are sequestered in anatomic sites other than the spleen, i.e., that the reason no tolerance was transferred was because the wrong cell types were transferred. [see also chapter II, In vivo fate of ^{125}I-HIgG(mPEG)$_{20}$ and ^{125}I-sham conjugated HIgG#1].

Mice (intact) receiving two widely separated dose of conjugate showed no features of an anamnestic response with respect to suppression, i.e., that the suppression induced would (a) appear more rapidly; (b) be more profound and (c) be of longer duration in comparison with animals that had received only one injection of the conjugate. Therefore this experiment could not compare the tolerant state in donor versus recipient mice.

In the case of intact mice receiving two widely separated

injections of conjugate the data obtained here could possibly be interpreted as due to the induction of memory B cells by the antigenic determinants present on the tolerogen which are restimulated upon re-exposure to tolerogen. (i.e., since it was shown previously that the tolerance induced by the conjugate was antigen specific then logically some antigenic determinant(s) must be recognised by the immune system. To the outside observer it appears that there is no antigenicity associated with the conjugate since its net effect is the absence or reduction of an immune response). Thus, there is a rapid expansion of B cells which counteract the suppressive effects of the second dose of tolerogen. It is possible that an increase in the dose of tolerogen and varying the time interval between administrations of conjugate may shift the equilibrium in favour of enhanced tolerance. Some evidence that this may indeed be the case is provided by the observations described previously that the administration of multiple injection of conjugate over a period of 14 days resulted in suppression despite daily immunisations.

In the case of the recipient mice which were immunised after receiving cells from donor mice given two widely separated injections of conjugate with transfer 6 days following the second injection, suppression was observed. However, this suppression was not as profound (4,913) compared with the groups receiving only one injection of conjugate on day 57 (974) of the experiment. It is apparent therefore that former group did not demonstrate anamnestic

features with respect to suppression induction. The transfer of splenic leukocytes into naive syngeneic recipients places the putative T suppressor cells and/or factors in an immunological environment where the host's unprimed HIgG specific Th, Ts or B cells may dominate, in contrast to administering two separate dose of conjugate to the same (intact) mouse where such cells are already primed.

Transfer of tolerance by subpopulations of splenic leukocytes from tolerised mice

Introduction

In order to characterise the cell type(s) responsible for transferable suppression splenic leukocytes from mice tolerised with 100 micrograms of HIgG(mPEG)$_{20}$ were fractionated into B cell enriched or surface Ig positive (sIg+) and into T cell enriched or surface Ig negative (sIg-) populations essentially according to the method of Mage et al (176). The ability of leukocytes, sIg+ and sIg- cell populations, derived from conjugate and PBS treated donors, to transfer tolerance was investigated.

Materials and Methods

Separation of sIg+ cells from sIg- cells was achieved as follows: Corning polystyrene tissue culture grade petri dishes (10 cm in diameter) were each incubated overnight at 4 ^{0}C with 5 ml of sheep anti-mouse hyperimmune serum (3 ml at 50 micrograms/ml per dish) which was kindly supplied by S. Krueger. Plates were washed three

times by swirling with PBS and then any unoccupied binding sites were blocked by incubating with a solution of BSA in PBS (5 ml at 0.1 g/ml) for 1 hour. Plates were again washed and spleen cell suspensions (prepared as described above) added to each plate (1 x 10^8 cells/plate). The plates were left at room temperature for 2 hours and given gentle manual swirling for 30 seconds, every 15 mins. At the end of the incubation period the non-adherent cells, (sIg-), were resuspended by swirling the plates, and removed. These non-adherent cells were then given a second cycle of absorption onto fresh coated petri dishes exactly as before. The plates were washed with PBS 5 times to remove any remaining non-adherent cells and 5 ml of MEM-Hepes added to each plate. The adherent cells (sIg+) were dislodged from the plates using a rubber policeman, then counted and their viability determined as described above. 2 x 10^7 sIg+ cells (or 3 x 10^7 sIg- cells) in 0.5 ml of MEM-Hepes were injected, i.v., into naive syngeneic recipients. The numbers of cells transferred were chosen so as to reflect the relative numbers of T and B cells present in a half spleen i.e., 5 x 10^7 leukocytes. Control mice received MEM-Hepes (0.5) ml only or cells from non tolerised donors i.e. those given PBS rather than conjugate on d -6. All groups were immunised the day following injection of cells and were bled 14 and 21 days post-immunisation.

Results

IgG1 titers are presented in table 10 and the means are plotted in

figure 9. Recipients of sIg- leukocytes derived from donors treated with conjugate showed suppression e.g., 2,011 for the average day 14 titer compared with an average day 14 titer of 18,096 for recipients of normal cells (i.e., cells derived from donor mice treated with PBS in place of conjugate). This is comparable (i.e., not statistically different) to the suppression observed in recipients of 5×10^7 leukocytes from tolerised mice i.e., an average day 14 titer of 3,135. Recipients of sIg+ leukocytes derived from tolerised donors showed reduced but significant suppression with an average day 14 titer of 13,859. The titers for transfer of MEM or PBS were not statistically different from each other. Suppression where observed, was maintained at day 21.

Discussion

Basten et al (129) and Doyle et al (131) have characterised the cell responsible for transferable suppression induced by HGG as a T cell. The data presented here are consistent with their findings in that the cell(s) mediating the tolerance induced by $HIgG(mPEG)_{20}$ are present in the sIg- or T cell enriched population. The tolerance transferring ability of the sIg- cell population does not appear to be due to antigen carry-over (table 11 group b)as at most 2.41 ng of antigen was carried over. This should be compared to 1 or more micrograms of $HIgG(mPEG)_{20}$ required to induce tolerance in donor mice.

The efficiency of the separation method was assessed by incubating

the ostensibly sIg- population with sheep erythrocytes coated with anti-Ig. It was found that less than 1% of cells in the sIg- population formed rosettes (data not shown).

Characterisation of cells mediating transferable suppression

Introduction

In order to further characterise the leukocyte population mediating transferable suppression the commercially produced (Cedar Lane, Canada) Lyt monoclonal antibodies anti-Lyt-1.2 and anti-Lyt-2.2 were used in conjunction with rabbit complement) to treat tolerant splenic leukocytes before injection into recipients. Rabbit serum provides a potent source of complement for cytotoxicity techniques utilising antisera to mouse lymphocytes. However, rabbit serum per se is toxic to mouse lymphocytes and is therefore absorbed out before use using agarose and mouse tissues.

Materials and Method

Tolerised or normal splenic leukocytes (obtained as described previously) were washed three times in MEM (1,200 rpm, 10 min, 4^0C) and the cell concentration adjusted to 1 x 10^7 cells per ml. Cells were incubated with either anti-Lyt-2.2 (final concentration 1:20) (177) or anti-Lyt-1.2 (final concentration 1:500) (178) or with MEM for 60 min at 4^0C. After pelleting the cells, they were resuspended in LO-Tox rabbit complement (Cedar Lane) (final concentration 1:10) (179) or in MEM and incubated for 60 min at 37^0C. The percent

cytotoxicity was measured by the trypan blue exclusion method. Dead cells were removed by centrifugation on Ficoll-Hypaque as above. Cells were washed x 3 in MEM and injected (5×10^7) i.v. into naive syngeneic recipients. Recipients were immunised the following day and bled 14 and 21 days post-immunisation.

Results

IgG1 titers are presented in table 15 and means are plotted in figure 13. Mice receiving normal leukocytes from PBS (i.e. non-tolerised) donors showed slight suppression (day 14 average of 18,128) compared with recipients who were given MEM in place of cells (day 14 average of 20,033). This non-specific effect has been observed on other occasions and may be due to perturbation of the homeostatic balance in the recipients by the injection of 5×10^7 cells. In order to take into account this non-specific suppression, the average titers of mice receiving normal cells was considered as 100% response for the purpose of calculating percent suppression.

Mice receiving tolerised cells treated only with MEM showed 80% suppression and those receiving tolerised cells treated with anti-Lyt-1.2 and complement showed 61% suppression. However, recipients of tolerised cells treated with anti-Lyt-2.2 and complement showed only 7% suppression. Recipients of tolerised cells treated with complement alone showed 69% suppression.

Table 15:
The effect of anti-Lyt 1.2/2.2 and complement on the transfer of of tolerance by splenic leukocytes from tolerised mice

			mean IgGl anti-hIgG titer (2 data points per mean)			
group	percent kill	C.I.	day 14	S.D.	IgGl titer day 21	S.D.
(a)*	4	-	3,156	346	4,106	771
(b)*	10	-	4,767	913	5,570	638
(c)*	26	10.0	7,272	745	7,946	91
(d)*	25	11.0	16,617	871	15,835	1,621
(e)*	-	-	20,033	535	21,811	395
(f)*	6		18,128	1,244	18,313	2,816
(g)	10	_	21,090	1,310	24,955	1,353
(h)*	16	7.0	17,983	71	20,269	3,680
(i)	18	9.0	23,949	1,480	22,833	3,999
(j)	-	-	23,539	781	23,594	211

5×10^7 splenic leukocytes derived from donor mice tolerised with 100 micrograms of HIgG(mPEG)20, i.v., were incubated with (a) MEM (b) complement only, (c) anti-Lyt-1.2 antibodies + complement or (d) anti-Lyt-2.2 antibodies + complement. These cells were then injected, i.v., into naive syngeneic recipients. Group (e) received MEM in place of cells.
5×10^7 splenic leukocytes derived from donor mice injected with PBS were incubated with the following: group (f) with MEM only (g) complement only, (h) anti-Lyt-1.2 antibodies + complement or (i) anti-Lyt-2.2 antibodies + complement. These cells were then injected, i.v., into naive syngeneic recipients. Group (j) received MEM in place of cells.

$$\text{cytotoxic index (C.I.)} = \frac{100\% \text{ cyt(Ab + C)} - \% \text{ cyt (C alone)}}{100 - \% \text{ cyt (C alone)}}$$

Figure 13; Legend
Effect of anti-Lyt 1.2 and anti-Lyt 2.2 plus C on suppressive activity of splenic leukocytes from tolerised mice.

5×10^7 splenic leukocytes derived from donor mice tolerised with 100 micrograms of $HIgG(mPEG)_{20}$ were incubated with one of the following: MEM, complement only, anti-Lyt 1.2 antibodies and complement or anti-Lyt 2.2 antibodies plus complement. These cells were then injected, i.v., into naive syngeneic recipients. A control group received MEM in place of cells.

All recipient groups were immunised one day after receiving cells or MEM. They were bled 14 and 21 days post-immunisation and their sera assayed for IgGl anti-HIgG antibodies

No cells = recipients injected with MEM only

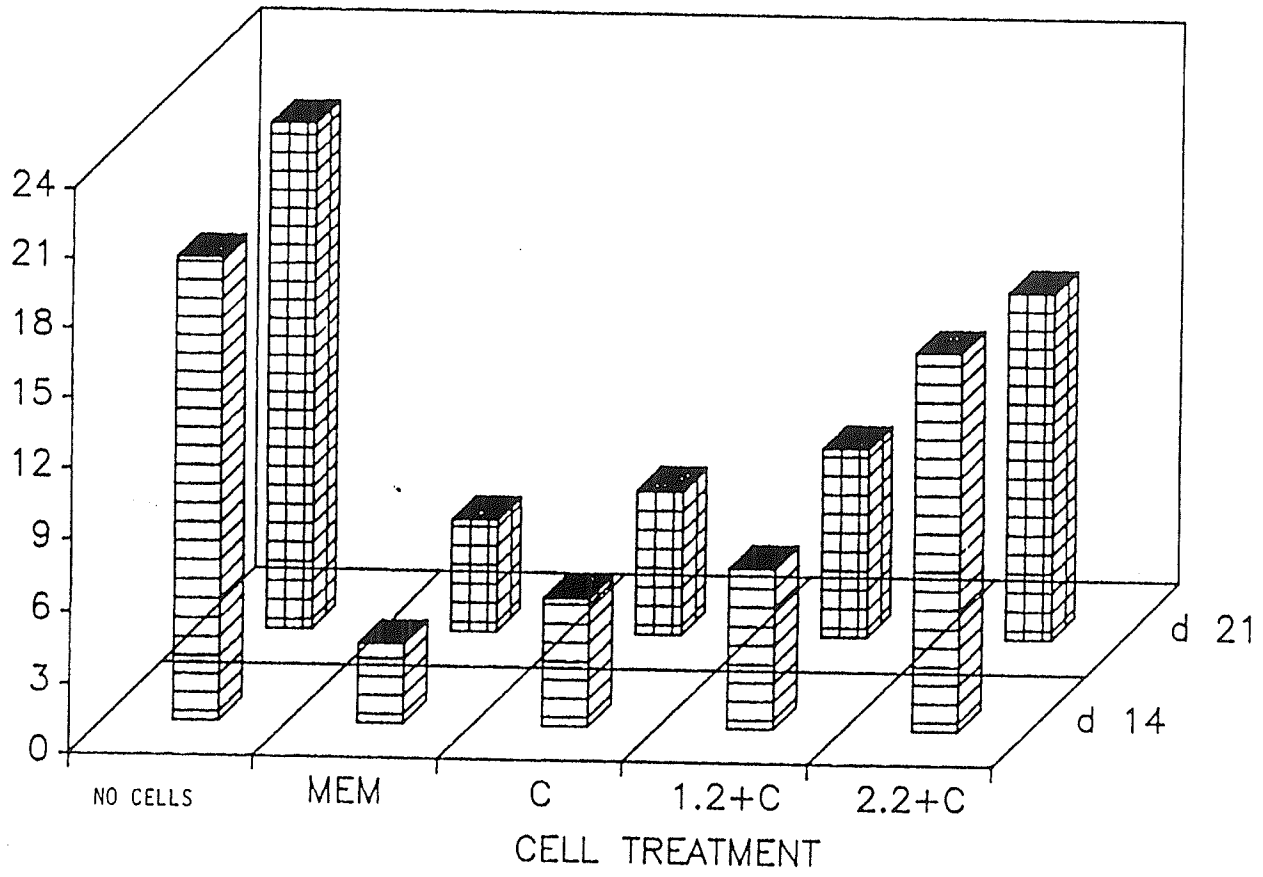
MEM = splenic leukocytes derived from donor mice injected with conjugate and incubated with MEM only prior to injection into recipients.

C = splenic leukocytes derived from donor mice injected with conjugate and incubated with complement only prior to injection into recipients.

1.2 + C = splenic leukocytes derived from donor mice injected with conjugate and incubated with anti-Lyt 1.2 antibodies and complement prior to injection into recipients.

2.2+C = splenic leukocytes derived from donor mice injected with conjugate and incubated with anti-Lyt 2.2 antibodies and complement prior to injection into recipients.

Figure 13: EFFECT OF ANTI-LYT 1.2 AND ANTI-LYT 2.2 PLUS C ON SUPPRESSIVE ACTIVITY OF SPLENIC LEUCOCYTES FROM TOLERISED MICE



Discussion

The cytotoxicity of Lyt antisera and complement vary with the haplotype and organ source of the lymphocytes under investigation. The mice used in this study (BDF1) are the F1 hybrids of C57BL/6 x DBA/2 parents, which are phenotypically Lyt-1.2,2.2 and Lyt-1.2,2.1 respectively. No data was available as regards killing of BDF1 cells by these commercial MAbs but both Lyt antisera are known to be capable of killing C57BL/6 lymphocytes (177,178). F1 hybrids between two inbred strains express histocompatibility antigens of both parental strains i.e., expression is codominant (123). Anti-Lyt-2.2 has a cytotoxic index (C.I., where C.I. = 100 x % (antibody + complement) - % cytotoxicity (complement alone) / 100 - % cytotoxicity (complement alone)) of only 5% for C57BL/6 spleen cells compared with 82% for thymocytes. In the case of anti-Lyt-1.2 in the same strain of mice the C.I. is 22% and 90% respectively.

In the present study a C.I. of 12% was recorded for anti-Lyt-1.2 with splenic leukocytes and 9% for anti-Lyt-2.2. Treatment of tolerant cells with anti-Lyt-2.2 plus complement had the greatest effect (i.e., reduced suppressive ability) with an average day 14 titer of 16,617 compared with an average day 14 titer of 3,156 for tolerant cells treated only with MEM. Treatment of tolerant cells with anti-Lyt-1.2 plus complement was less effective at reducing suppression although the reduction was significant with a day 14 average titer of 7,272 (even though it had a C.I. of 12 for anti-Lyt-1.1 compared with a C.I. of 9 for anti-Lyt-2.2). Treatment

with anti-Lyt-2.2 was significantly different from treatment with anti-Lyt-1.1. Treatment of tolerant splenic leukocytes with complement alone however also resulted in some non-specific cytotoxicity (13%) and this reduced the suppressive effect of the leukocytes (average day 14 titer of 4,767 compared with 3,156 for untreated tolerant cells although this was not statistically significant.

Treatment of normal cells i.e. splenic leukocytes derived from donor mice given PBS in place of conjugate, with the MAbs and/ or complement had no effect on titers.

Basten et al (143) showed that suppression of the anti-DNP plaque forming colony (PFC) response by purified tolerant T cells was completely abrogated by anti-Lyt-2.1 but not by anti-Ly-1.1. The Lyt-2 phenotype of the HGG specific suppressor cell was also demonstrated by Vadas et al (132) and by Taniguchi and Miller (133). This is in contrast to Dorf et al (91) who showed that the initial cell (in a T cell circuit) mediating transferable suppression was Lyt-1 positive using the DNP system. This difference in phenotype may reflect the different nature of the two antigens involved i.e., DNP is a hapten in contrast with HGG which possesses multiple haptenic determinants.

Transfer of suppression by leukocytes and subpopulations of leukocytes derived from donor mice tolerised with $HIgG(mPEG)_{17}$

Introduction

It was shown previously that the conjugate $HIgG(mPEG)_{17}$ (synthesised from a different human myeloma protein (HIgG#2) than that used to synthesise the conjugate $HIgG(mPEG)_{20}$) was tolerogenic when given i.v. at a dose of 100 micrograms per mouse. Transfer experiments analogous to those previously described for $HIgG(mPEG)_{20}$ were carried out using $HIgG(mPEG)_{17}$ as the tolerising agent. In addition the effect on tolerance was investigated by varying the number and type of cells transferred in comparison with equivalent populations of normal cells (i.e., cells derived from donor mice treated with PBS in place of conjugate). By diluting various populations of tolerant leukocytes and injecting them into naive syngeneic recipients, the cell(s) responsible for mediating transferable suppression were further characterised.

Materials and Methods

Splenic leukocytes, sIg+ and sIg- enriched populations were obtained as described previously. The sIg- cell population was further fractionated into receptor HIgG+ (rHIgG+) and receptor HIgG- (rHIgG-) populations as follows: Enrichment for sIg-/rHIgG+ cells was carried out essentially as described by Mage et al (176) and by Taniguchi and Miller (133). Plastic petri dishes were coated with 3 ml of PBS containing DEAE-purified HIgG (Cappel) at a concentration

of 1 mg/ml and incubated overnight at 4^0C. The plates were washed 5 times with PBS and sIg- splenic leukocytes, obtained as described previously, added (2×10^7 cells per plate). After incubation at RT for 1 hr, the plates were swirled, the non-adherent cells (sIg-/rHIgG-) removed and washed twice with MEM before injection into naive syngeneic recipients. The plates were then incubated for 15 min at 4^0C and the adherent cells (sIg-/rHIgG+) removed from the plate. The cells were washed twice in MEM before injection into naive syngeneic recipients. Recipients were given 5×10^a leukocytes (where a = 7, 6, 5 or 4), 3×10^a sIg+ leukocytes, 2×10^a sIg- leukocytes, 2×10^a sIg-/rHIgG- leukocytes or 2×10^a sIg-/rHIgG+ derived from donor mice treated with conjugate or PBS. Recipients were immunised the following day and bled 14 and 21 days post-immunisation.

Results

IgG titers are presented in table 16 and average percent suppression vs log of the number of cells transferred is plotted in figure 14 (with the exception of the data for sIg-/rHIgG- cells). [Please note that percent suppression is calculated using the average day 14 titers for a given cell population/cell number and considers the average day 14 titer for the equivalent number of normal cells (i.e., cells derived from donor mice treated with PBS in place of conjugate) as 100 %].

Transfer of sIg+ cells from tolerised donors resulted in no

Legend to Table 16:

Donor mice received 100 micrograms of HIgG(mPEG)17, i.v., or PBS and were killed 6 days later and their splenic leukocytes (groups (1-4)) were transferred into naive syngeneic recipients, i.v.. Each recipient was given 5×10^{x} where x = 7,6,5 or 4

Group (5) received MEM only. Groups (6-9) received a sIg+ fraction of splenic leukocytes obtained by separation on sheep anti-mouse Ig coat -ed petri dishes (3×10^{x}). Groups (10-13) received sIg- leukocytes obtained as in groups (6-9) above (2×10^{x}). Groups (14-17) received sIg-/sHIgG- leukocytes obtained by further fractionating the sIg- cell -s obtained as in groups (10-13) above by incubation on petri dishes coated with HIgG (2×10^{x}). Groups (18-21) received sIg-/sHIgG+ cell -s obtained as in groups (14-17) above (2×10^{x}).

The remaining groups are controls for each of the above populations, i.e. these recipients were given identical numbers/populations of cell -s but the leukocytes were derived from mice injected with PBS only. For example groups (22-25) are the controls for groups (1-4) and group -s (26-29) are the controls for groups (6-9) etc.

All recipients were immunised one day after receiving leukocytes or MEM with 50 micrograms of haIgG#2. All mice were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG anti -bodies.

Group	Number of data points per mean
(1)	4
(2-4)	2
(5)	4
(6-18)	2
(19)	1
(20-21)	2
(22)	4
(23-41)	2

Table 16:
Transfer of HIgG(mPEG)17 induced suppression as a function of cell population and number.

day -6 day 0

injection of donor	type of cell received	# of cells received	Mean IgG day 14	S.D.	anti-HIgG titer day 21	S.D.
conjugate*	leukocytes	5×10^7	4,139	1,415	5,245	827
(2) "	"	5×10^6	16,459	1,549	18,174	2,152
(3) "	"	5×10^5	22,506	1,685	21,206	1,892
(4) "	"	5×10^4	25,823	1,662	24,153	2,181
(5) "	MEM	-	22,359	1,832	20,978	1,215
(6) "	sIg+	3×10^7	28,296	1,923	26,247	2,254
(7) "	"	3×10^6	27,008	1,919	24,632	3,280
(8) "	"	3×10^5	25,559	1,519	24,528	3,434
(9) "	"	3×10^4	24,738	3,639	25,118	3,927
(10)* "	sIg-	2×10^7	2,758	1,121	2,438	179
(11)* "	"	2×10^6	7,807	1,625	7,172	1,015
(12)* "	"	2×10^5	11,311	1,524	11,311	1,491
(13) "	"	2×10^4	17,613	2,758	18,698	1,136
(14)* "	sIg-/sHIgG	2×10^7	6,697	1,412	7,221	2,517
(15)* "	"	2×10^6	11,740	2,099	11,155	1,824
(16) "	"	2×10^5	20,060	818	20,768	2,507
(17) "	"	2×10^4	22,577	3,425	23,773	1,235
(18)* "	sIg-/sHIgG	2×10^7	1,411	771	1,554	689
(19)* "	"	2×10^6	1,195	-	2553	-
(20)* "	"	2×10^5	1,479	675	1,515	6,338
(21)* "	"	2×10^4	13,759	1,590	16,108	2,197
(22) PBS	leukocytes	5×10^7	21,173	2,475	20,553	1,880
(23) "	"	5×10^6	23,770	1,570	21,948	542
(24) "	"	5×10^5	20,851	1,199	19,860	432
(25) "	"	5×10^4	23,113	2,045	23,005	1,396
(26) "	sIg+	3×10^7	23,519	2,007	22,073	2,722
(27) "	"	3×10^6	22,613	1,441	22,607	1,488
(28) "	"	3×10^5	22,234	1,881	22,808	438
(29) "	"	3×10^4	23,492	1,660	23,510	2,129
(30) "	sIg-	2×10^7	23,770	1,720	23,698	1,301
(31) "		2×10^6	22,881	3,864	22,076	2,778
(32) "	"	2×10^5	19,323	465	20,399	2,246
(33) "	"	2×10^4	21,772	1,095	21,944	2,432
(34) "	sIg-/sHIgG	2×10^7	21,946	2,911	22,229	337
(35) "	"	2×10^6	20,054	2,956	22,312	1,194
(36) "	"	2×10^5	25,118	2,013	23,448	303
(37) "	"	2×10^4	24,511	2,871	22,746	3,816
(38) "	sIg-/sHIgG	2×10^7	20,438	308	23,746	1,144
(39) "	"	2×10^6	20,186	253	19,055	863
(40) "	"	2×10^5	24,055	701	23,602	66
(41) "	"	2×10^4	23,038	4,135	22,396	72

Figure 14: Legend
Percent suppression vs number and type of tolerised cells transferred

Unfractionated leukocytes = Donor mice received 100 micrograms of HIgG(mPEG)$_{17}$, i.v., or PBS and were killed 6 days later. Their splenic leukocytes (unfractionated leukocytes) were transferred into naive syngeneic recipients, i.v.. Each recipient was given 5 x 10^x cells, where x = 7, 6, 5, or 4.

SIg- = splenic leukocytes obtained as described above were fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. Recipient mice were injected, i.v., with 2 x 10^x cells, where x = 7, 6, 5, or 4.

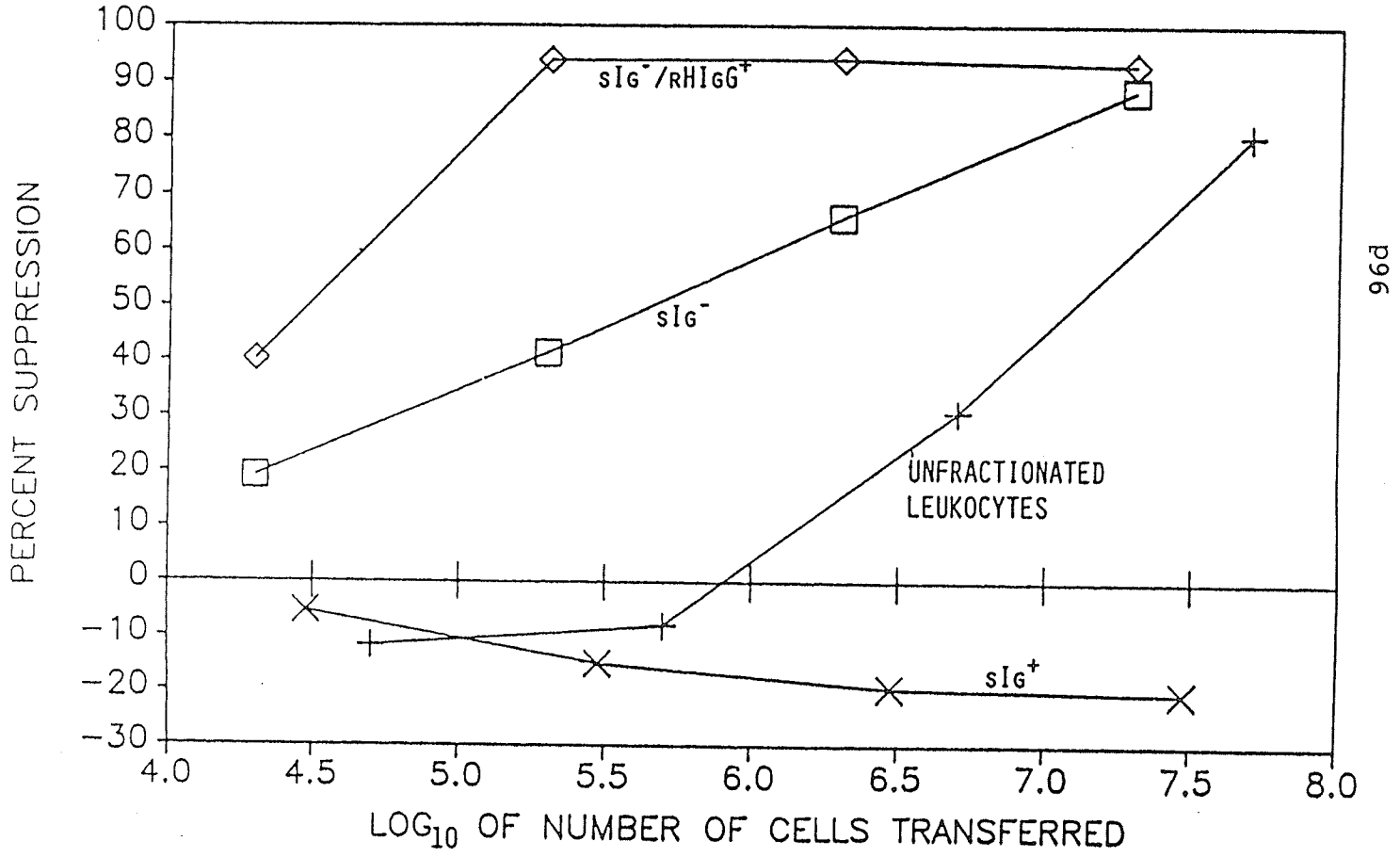
SIg+ = those cells which adhered to the petri dishes as described above. Recipient mice were injected, i.v., with 2 x 10^x cells, where x = 7, 6, 5, or 4.

sIg-/rHIgG+ = sIg- cells obtained as described above were further fractionated by incubation on petri dishes coated with HIgG. The adherent population = sIg-/rHIgG+. Recipient mice were injected, i.v., with 2 x 10^x cells, where x = 7, 6, 5, or 4.

A control group of recipient mice received MEM in place of cells. All recipient groups were immunised, i.p. with 50 micrograms of haIgG#2 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies.

Percent suppression = 100 - (the mean day 14 titer for a given cell population/number expressed as a percentage of the mean day 14 titer of recipients of the same cell population/number of normal cells, i.e., cells derived from donor mice given PBS only). Note that numbers of cells are plotted as corresponding logarithms.

Figure 14: PERCENT SUPPRESSION vs NUMBER AND TYPE OF TOLERISED CELLS TRANSFERRED



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suppression at any dilution compared with equivalent numbers of sIg- cells derived from normal donors (day 14 averages of 28,296 compared with 23,519; 27,008 compared with 22,613; 25,559 compared with 22,234 and 24,738 compared with 23,492).

Transfer of 5 x 10^7 unfractionated leukocytes derived from tolerised donors resulted in suppression compared with equivalent numbers of normal unfractionated leukocytes (day 14 average titer of 4,139 compared with 21,173 or 81 % suppression). However, the suppressive effect fell rapidly when the number of cells was reduced by a factor of ten (16,459 compared with 23,770 or 31% suppression).

Transfer of 2 x 107 sIg- leukocytes derived from tolerised donors resulted in suppression compared with equivalent numbers of normal cells (day 14 average titer of 2,758 compared with 23,770 or 88% suppression). The suppressive effect diminishes as the number of sIg- cells is reduced but at a slower rate than that for unfractionated tolerant leukocytes e.g., 2 x 10^5 sIg- cells can induce 42% suppression whereas 5 x 10^5 unfractionated leukocytes show no suppressive effect.

The most potent suppressive ability was observed in the sIg-/rHIgG+ cell population which showed > 90 % suppression despite dilution from 2 x 10^7 down to 2 x 10^5 cells.

The sIg-/HIgG+ cell population showed suppressive potency less than that of sIg- cells but greater than that of unfractionated leukocytes.

Discussion

The suppressive potency of the tolerant cell populations may be ranked as follows: sIg-/rHIgG+ > sIg- > sIg-/rHIgG- > unfractionated leukocytes > sIg+ cells. It might be expected that the sIg-/rHIgG- cells should be at least as suppressive as sIg- cells but instead they were observed to be less potent at transferring suppression. However, the incubation of sIg- cells with HIgG appears to remove some suppressive cells which adhere to the HIgG coated dish during the fractionation procedure. As HIgG was used to coat the petri dishes, it is possible that cells bearing Fc receptors could have been removed along with HIgG-specific cells. If the suppressor cells were FcR positive, their enrichment may have been FcR-dependent in addition to being HIgG dependent. However, a counter argument to this is that the sIg- incubated on the HIgG coated petri dishes, were themselves selected by incubation (twice) of splenic leukocytes on sheep anti-mouse Ig coated dishes during which most FcR+ cells should have been removed.

Incubation on antigen (HIgG) coated petri dishes failed to deplete all T suppressor cells (the latter have been shown to be antigen specific). The panning technique relies upon the settling out of cells from suspension in the bulk volume of MEM, onto a HIgG coated region of the petri dish surface that is not already occupied by other cells (the latter being specifically or possibly non-specifically bound).

Okumura et al (180) have shown that I-J+ suppressor T cells belong

to a FcR- subset.

Taniguchi and Miller (133) reported a 100 fold enrichment of sIg- cells (a similar degree of enrichment was obtained in the present work) by allowing the sIg- cells to adhere to HGG-coated petri dishes. By contrast the cells not adhering to the HGG coated dishes were not suppressive. Similar results were reported by Maoz et al (180) who enriched antigen-specific suppressor and helper cells on antigen-derivatised collagen gel-coated plates.

Chapter IV

Examination of the properties of freeze/thaw extracts of tolerised leukocytes

Introduction

In order to further study the suppression induced by sIg- cells derived from donor mice tolerised with HIgG$(mPEG)_n$ conjugates, cell extracts were obtained by subjecting these cells to freezing and thawing. Several investigators have reported that factors extracted from suppressor T cells can suppress the immune response at various points in suppressor circuits. Tada et al (182) have described an antigen specific factor extracted from thymocytes and from spleen cells of rats hyperimmunised with a carrier protein. Administration of this cell extract into syngeneic recipients abrogated an ongoing IgE antibody response to a hapten coupled to the original carrier. The factor was shown to (a) bind antigen; (b) possess no conventional Ig determinants; (c) inhibit antibody responses in an antigen specific manner and (d) to be a protein of molecular weight < 70,000 Daltons. Using a similar murine system, the same group showed that the suppressive factor derived from hyperimmunised mice had the following characteristics: (a) bore I-J subregion coded determinants (183), (b) acted on antigen primed Lyt-1+2+ T cells to generate the suppressor-effector T cell and (c) showed I-J restriction in order to elicit effective suppression in its interaction with effector T suppressor cells (184). Similar antigen specific, genetically

restricted suppressive factors have been described by Kapp et al (185-187), Waltenbaugh et al (188) and Theze et al (189,190). using GAT and GT as antigens. In addition, secreted suppressive factors (i.e., produced in vitro) have been described by Yamauchi et al (191,192).

PART I: The transfer of suppression by F/T_{20} extract

Transfer of tolerance by freeze/thaw (F/T) extract of $HIgG(mPEG)_{20}$ tolerised sIg- splenic leukocytes

Introduction

It was shown previously that 2×10^7 sIg- splenic leukocytes derived from donor mice tolerised with 100 micrograms of $HIgG(mPEG)_{20}$ were capable of transferring suppression when given i.v. into naive syngeneic recipients. The experiments described in this section investigated the ability of freeze/thaw extracts (F/T) of sIg- splenic leukocytes derived from $HIgG(mPEG)_{20}$ tolerised donor mice (denoted as F/T_{20}) and from $HIgG(mPEG)_{17}$ tolerised donor mice (denoted as F/T_{17}) to transfer suppression into naive syngeneic recipients. An F/T extract was also made from sIg- splenic leukocytes derived from normal donor mice i.e., mice that had received PBS in place of conjugate (denoted as F/T_{normal}).

Materials and Methods

Surface Ig- cells derived from donor mice tolerised with 100 micrograms of HIgG$(mPEG)_{20}$ or from mice treated with PBS in place of conjugate 6 days prior to sacrifice, were obtained as described previously. An aliquot of these cells was injected, i.v. into naive syngeneic recipients at a dose of 2×10^7 cells per mouse in 0.5 mls of MEM. The rest of the cells were then suspended in MEM-Hepes at 4×10^7 cells per ml and frozen at -70^0C then thawed at 37^0C, three times in succession. The resulting lysate was centrifuged at 10,000 rpm for 10 mins in order to remove cell debris, and the supernatant (i.e. the F/T_{20} extract) was removed. Recipient mice were injected with 0.5 mls i.e., 2×10^7 cell-equivalents of F/T_{20} extract.

In order to estimate the molecular weight of any suppressive factor(s) present in the F/T extract an aliquot of F/T_{20} was passed through an Amicon XM 100A membrane (molecular weight cut-off = 100 kD) and both the retentate (i.e., material > 100 kD) and the filtrate (i.e., material < 100 kD) injected i.v. into naive syngeneic recipients (2×10^7 cell equivalents). Recipient mice were immunised the following day and bled 14 and 21 days post-immunisation. Control groups were immunised with AOA in place of haIgG.

Results

IgG1 titers are presented in table 17 and their means are plotted in figure 15 with the exception of the AOA control groups. Recipients of tolerant sIg- cells showed suppression with a day 14 average titer

Table: 17
Transfer of tolerance by freeze/thaw (F/T) extract of tolerised splenic sIg- leukocytes

			Mean IgG1 anti-HIgG$^{\mp}$ titer (2 data points per mean)			
group	day -6	day 0	day 14	S.D.	day 21	S.D
(a)*	conj.	sIg-	2,022	91	2,815	562
(b)	PBS	"	10,011	1,565	10,960	2,065
(c)*	conj.	F/T20	1,070	976	1,987	1,945
(d)*	PBS	F/Tn	8,790	2,447	9,203	-
(e)	conj.	F/T20>100	9,558	1,720	11,271	2,758
(f)	PBS	F/Tn>100	10,031	1,662	11,051	1,816
(g)*	conj.	F/T20<100	4,500	1,370	5,373	1,433
(h)*	PBS	F/Tn<100	8,097	1,578	8,250	778
(i)	"	MEM	14,301	1,160	14,893	1,373
(j)	"	"	9,276	2,223	8,061	71
(k)	conj.	F/T20	10,423	1,701	10,165	2,911
(l)	"	F/Tn	7,139	936	7,505	2,411

Donor mice received 100 micrograms of HIgG(mPEG)20 conjugate, i.v. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- cells using petri dishes coated with sheep anti-mouse Ig (groups (a and b)). A freeze/thaw extract of these cells was produced and given to groups (c) and (k). This F/T20 ex -tract was itself fractionated by passage through an Amicon X-100 -A membrane (molecular weight cut-off = 100 kD). This produced two fractions: F/T20<100 = that fraction which passed through the membrane and F/T20>100 = that fraction which did not pass through the membrane. Group (g) received F/T20<100 and group (e) received F/T20>100.

Control groups received sIg- leukocytes derived from donor mice that had received PBS in place of HIgG(mPEG)20. A similar freeze/thaw extraxct was prepared (F/Tn given to group (d)) and fractionated (F/Tn<100 given to group (h) and F/Tn>100 given to group (f)).

Other control groups received no cells or extracts but only MEM. MEM was used throughout as the diluent.

All the above groups were immunised one day following injection of cells, extracts or MEM, with 10 micrograms of haIgG#1, i.p..

$^{\mp}$To examine the antigenic specificity of any transferred tolerance mice that had received MEM, F/T20 or F/Tn were immunised with AOA (50 micrograms, i.p.) instead of haIgG#1 (groups j, k & l).

All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Figure 15: Legend
Transfer of tolerance by freeze/thaw (F/T) extract of tolerant splenic Ig- leukocytes.

A = Donor mice received 100 micrograms of $HIgG(mPEG)_{20}$, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. These cells were transferred, i.v., into naive syngeneic recipients.
B = Donor mice received PBS only, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. These cells were transferred, i.v., into naive syngeneic recipients.
C = A freeze/thaw extract of the sIg- cells obtained as described in (A) above was transferred, i.v., into naive syngeneic recipients.
D = A freeze/thaw extract of the sIg- cells obtained as described in (B) above was transferred, i.v., into naive syngeneic recipients.
E = A freeze/thaw extract of the sIg- cells obtained as described in (A) above was further fractionated by passage through an Amicon X-100A membrane (molecular weight cut=off = 100 kD). The fraction that failed to pass through the membrane (F/T_{20}>100 kD) was transferred, i.v., into naive syngeneic recipients.
F = A freeze/thaw extract of the sIg- cells obtained as described in (B) above was further fractionated by passage through an Amicon X-100A membrane (molecular weight cut=off = 100 kD). The fraction that failed to pass through the membrane (F/T_{normal}>100 kD) was transferred, i.v., into naive syngeneic recipients.
G = The fraction of freeze/thaw extract that did pass through the membrane described in (E) above (F/T_{20}<100 kD) was transferred, i.v., into naive syngeneic recipients.
H = The fraction of freeze/thaw extract that did pass through the membrane described in (F) above (F/T_{normal}<100 kD) was transferred, i.v., into naive syngeneic recipients.
I = A control group of recipients received MEM only.

All recipient groups were immunised, i.p. with 10 micrograms of haIgG#1 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

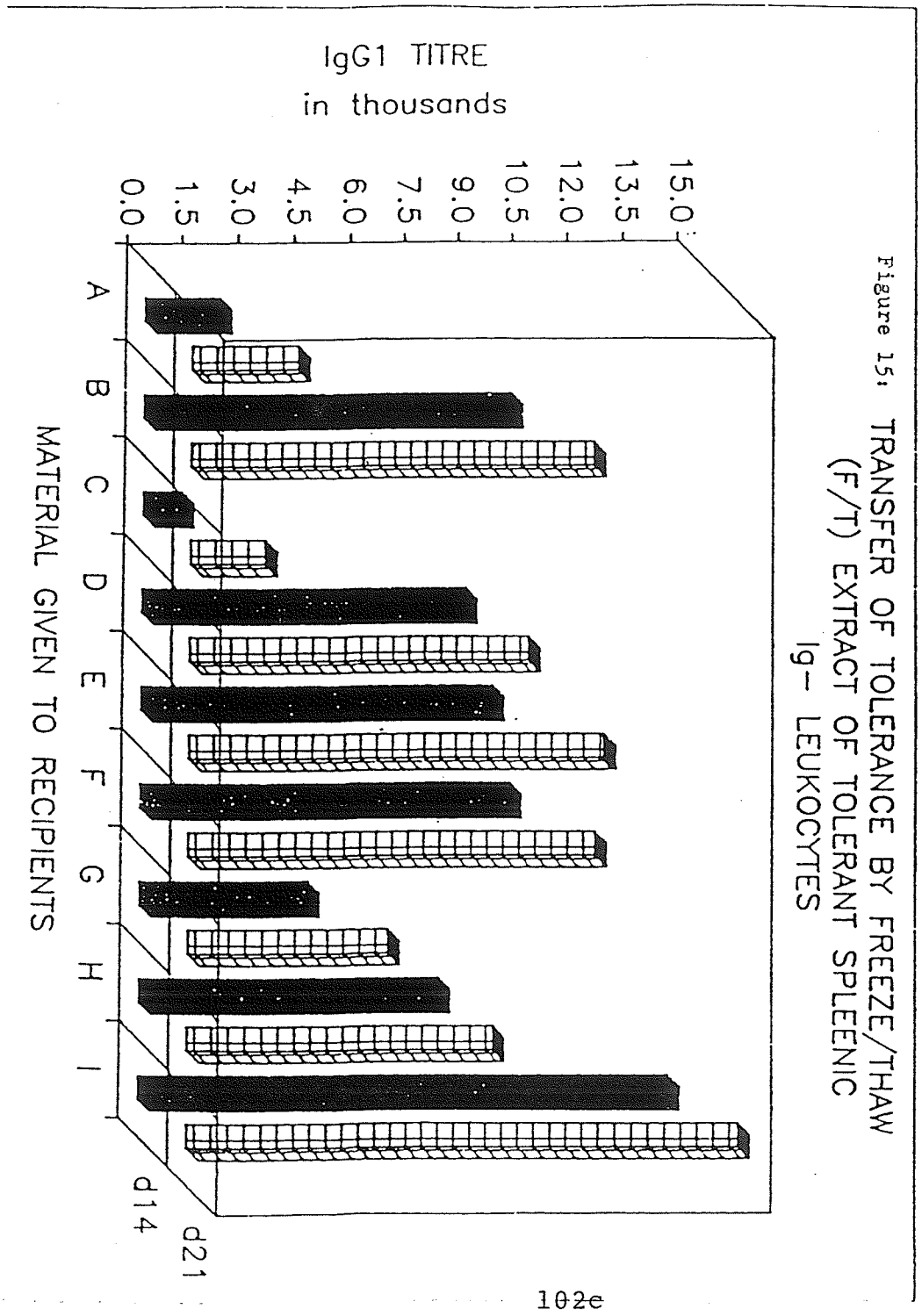


Figure 15: TRANSFER OF TOLERANCE BY FREEZE/THAW (F/T) EXTRACT OF TOLERANT SPLEENIC Ig- LEUKOCYTES

of 2,022 compared with 10,011 for recipients of normal sIg- cells. Recipients of F/T_{20} extract also showed suppression with an average day 14 titer of 1,070 compared with 14,301 for recipients of MEM.

Fractionation of the F/T_{20} extract into aliquots of < 100 kD and > 100 kD resulted in average day 14 titers of 4,500 (69% suppression) and 9,558 (33% suppression) respectively compared with 14,301 for recipients of MEM, suggesting that the suppressive moiety present in F/T_{20} may have a molecular weight of < 100 kD.

Recipients of normal sIg- cells or of F/T_{normal} showed some suppression compared with recipients of MEM with day 14 average titers of 10,011 (30% suppression), 8,790 (39% suppression) and 14,301 respectively. Fractionation of F/T_{normal} into aliquots of < 100 kD and > 100 kD resulted in average day 14 titers of 8,097 (43% suppression) and 10,031 respectively.

Recipients of F/T_{20} which were immunised with AOA in place of haIgG showed no suppression when compared with recipients which had been given MEM in place of F/T extract and immunised with AOA (day 14 average anti AOA titers of 10,423 and 9,276 respectively). Recipients of F/T_{normal} that were immunised with AOA in place of haIgG showed an average day 14 titer of 7,139 compared with 9,276 for recipients which had been given MEM in place of F/T extract and immunised with AOA.

Discussion

F/T_{20} is able to induce specific suppression (93%) compared with

86% suppression observed in recipients of 2×10^7 sIg- leukocytes derived from recipients of the conjugate. Surface Ig- leukocytes derived from normal donors (those that were given PBS in place of conjugate) also showed some suppression (30%).

The occasional observation of suppressive effects by normal leukocytes in transfer experiments has been alluded to previously. If the data is paired for tolerized versus normal mice for each group, i.e., titer for group PBS treated group - titer for conjugate or extract treated group/ titer for PBS treated group x 100; then the following percentages are obtained: 80% for sIg- leukocytes; 88% for F/T_{20}; 4% for the F/T_{20} fraction > 100 kD; and 44% for the F/T_{20} fraction < 100 kD.

Using these revised values it appears as if F/T_{20} and tolerant sIg- leukocytes are both capable of transferring suppression. However, the molecular weight fractionation data are inconclusive. Further transfer experiments using F/T extracts derived from mice tolerised with $HIgG(mPEG)_{20}$ and with $HIgG(mPEG)_{17}$ and with F/T extract derived from non-tolerised i.e., PBS treated mice, are described below.

It is apparent that normal (control) mice show some suppression when used as donors in transfer experiments using either their intact splenic leukocytes or freeze/thaw extracts of the latter. The degree of this effect is not consistant between experiments. However, mice treated with OA do not exhibit this effect; this may be due to the special nature of immunoglobulins [see chapter 1, The Special Nature of Immuoglobulins as Antigens]. This effect could be connected with

innate (pre-existing) regulatory networks within the naive mouse which serve to control autochthonous immunoglobulin. In terms of the model of idiotype networks, the naive mouse contains anti-autochthonous IgG clones and clones reactive to these anti-autochthonous clones et.c. [see chapter 1, Idiotype/anti-idiotype Interactions]. There are sets of clones that are reactive against isotypic, allotypic and idiotypic determinants present on autochthonous Ig molecules. (Presumably there are also sets of clones reactive with the carbohydrate moiety attached, post-translation, to Ig molecules). Entry of xenogeneic IgG, in certain physico-chemical forms, perturbs these clones with the net result of tolerance induction. In naive mice (controls) used as donors in transfer experiments, the leukocytes (or freeze/thaw extracts containing putative suppressor factor) themselves perturb the host's (recipient's) immune system with a net outcome similar to administration of tolerogen to the latter, i.e., suppression albeit not so profound. The strain of mouse used in the present work (BDF_1) are the F_1 product of C57BL/6 x DBA/2 inbred strains. Thus, any individual BDF_1 mouse is not necessarily homozygous at all loci with another individual. Recipient BDF_1 mice can thus recognise biologic materials derived from donor (naive) BDF_1 mice as non-self.

Tolerogen carry-over during transfer of tolerance by F/T_{20}

Introduction

In order to investigate the possibility that the suppressive

effects of F/T_{20} were due to carry-over of tolerogen, F/T_{20} derived from donor mice tolerised with $^{125}I\text{-}HIgG(mPEG)_{20}$ was given to recipients and the amount of conjugate associated with various leukocyte populations and F/T extracts estimated from the radioactivity counts; this quantity was compared with the minimum quantity of conjugate required to induce tolerance in the intact mouse.

Materials and Methods

The experimental protocol was identical to that previously described in chapter 3 for tolerogen carry-over by intact cells. The F/T extract was derived as described previously.

In order to compare the tolerizing ability of F/T_{20} with $HIgG(mPEG)_{20}$, the extract was injected into naive syngeneic recipients. Other groups of mice were given 0.01, 0.1 or 1 micrograms of $HIgG(mPEG)_{20}$, i,v, and immunised 6 days later. Recipients of F/T_{20} were immunised one day after administration of extract. All groups of mice were bled 14 and 21 day post-immunisation.

Results

In order to facilitate comparison the data are presented together with that for intact leukocytes in tables 11 and 12 (see chapter 3). Table 11 shows that an average of 0.46 ng of tolerogen was associated with 2×10^7 cell equivalents of F/T_{20}.

The radiolabelled extract was injected into naive syngeneic recipients which were immunised the following day. The IgG1 titers

for these recipient mice are presented in table 12 (see chapter 3). The radiolabelled F/T_{20} was able to transfer suppression with an average day 14 titer of 2,427 compared with an average day 14 titer of 21,162 for mice receiving MEM in place of extract.

Mice receiving 1 microgram of conjugate had an average day 14 titer of 16,402 compared with 19,410 and 18,621 for mice receiving 0.1 and 0.01 micrograms respectively.

Discussion

The amount of tolerogen apparently associated with the extract was approximately 100 - 1,000 fold less than the amount of conjugate required to directly tolerise a mouse (i.e., 0.46 ng and 1 - 10 micrograms respectively).

Radioiodination of antigens and tolerogens has been widely used in experiments similar to those described here to demonstrate that transferable suppression is not due to carry-over of antigen or tolerogen. However such experiments do not rule out the transfer of suppression by a `supertolerogen' i.e., some derivative of the tolerogen that has been physically altered in some way to produce a suppressorgenic substance different than the original tolerogen. These derivatives or the original tolerogen may have undergone de-iodination in vivo; thus the radioactivity levels associated with various cell populations or extracts may be due to free radioactive iodine or radioiodinated fragments of the tolerogen rather than the tolerogen per se.

Another point that must be considered is, how representative of the general population of tolerogen molecules are the radioiodinated derivatives? In the present study the cell populations and F/T extracts that were derived from donor mice given radioiodinated tolerogen, were tested in vivo to confirm that they exhibited tolerogenic properties similar to mice given non-radioiodinated conjugate.

Experiments utilising double radioactive labels e.g., the use of tritiated mPEG in molecules of the type: ^{125}I-HIgG(^{3}H-mPEG)$_n$ may help to resolve these problems (see chapter V).

Transfer of suppression by F/T_{20} extract of sIg- tolerised splenic leukocytes

Introduction

It was shown previously that F/T_{20} extract was capable of transferring suppression to the same degree as intact tolerised sIg- leukocytes. However the non-specific suppression induced by normal sIg- leukocytes and the F/T_{normal} extract derived from them, made interpretation of results difficult. Therefore, in order to clarify these observations the transfer of tolerance by F/T_{20} and F/T_{normal} was compared in a number of separate experiments.

In addition, F/T extracts derived from tolerised splenic leukocytes that had been treated with anti-Lyt monoclonal antibodies plus complement were examined for their tolerogenic properties.

Materials and Methods

F/T extracts were made as described previously; F/T_{20} being obtained from 2×10^7 sIg- leukocytes (or 5×10^7 unfractionated leukocytes in the case of the F/T_{20} obtained from cells treated with anti-Lyt-1.2 plus complement, anti-Lyt-2.2 plus complement or complement alone: see chapter 3) derived from donor mice given 100 micrograms of $HIgG(mPEG)_{20}$, i.v., 6 days prior to sacrifice and F/T_{normal} being obtained from the same number of sIg- leukocytes derived from donor mice given PBS, i.v., 6 days prior to sacrifice. The F/T extracts or MEM were injected, i.v., into naive syngeneic recipients which were immunised the following day and bled 14 and 31 days post-immunisation. Control groups were immunised with AOA in place of HaIgG.

Results

IgG1 titers are presented in table 18 and their means are plotted in figure 16, lower section (AOA control titers are not plotted). [Please note that the other graph showing the transfer of suppression by F/T_{17}, is presented on the same page in order to facilitate comparison between extracts derived from donor mice tolerised with $HIgG(mPEG)_{20}$ (lower section) and $HIgG(mPEG)_{17}$ (upper section). The graph in the upper section is described later].

The average day 14 titer for recipients of F/T_{20} is 3,222 compared with 19,315 for recipients of MEM. Recipients of F/T_{normal} had an average day 14 titer of 16,058 which is less than that of recipients

Table 18:
Transfer of tolerance by F/T(20)

group	donor	recipi-ent	Mean IgG1 anti-HIgG titer (groups d and e = anti-OA titer)			
	d -6	d 0	day 14	S.D.	day 21	S.D.
(a)	-	MEM	19,315	2,907	21,423	2,315
(b)	PBS	F/Tn	16,058	817	17,767	796
(c)*	conj	F/T20	3,222	2,003	3,165	1,491
(d)	-	MEM	11,182	2,205	11,719	2,887
(e)	conj	F/T20	11,902	1,369	11,490	2,135
(f)*	"	F/T20lyt1	7,719	1,180	8,884	1,109
(g)*	"	F/T20lyt2	13,879	1,561	13,272	3,859
(h)*	"	C	6,548	2,045	6,892	224

Groups of mice were injected with 100 micrograms of HIgG(mPEG)20 or PBS, i.v. and killed 6 days later. Their splenic leukoctytes were fractionated into sIg- cells by incubation on petri dishes coated sheep anti-mouse Ig. A freeze/thaw lysate of these cells was then injected i.v. into naive syngeneic recipients. Other mice received MEM only (groups (a) and (d). Group (f) received lysate derived from HIgG(mPEG)20 treated donors whose unfractionated splenic leukocytes had been incubated with anti-Lyt 1.2 + complement prior to injection into recipients. Group (g) received lysate derived from HIgG(mPEG)20 treated donors whose unfractionated splenic leukocytes had been incub-ated with anti-Lyt 2.2 plus complement. Group (h) received lysate derived from HIgG(mPEG)20 treated donors whose unfractionated splenic leukocytes had been treated with complement only.

Groups (a) to (c) and (f) to (h) were immunised one day following receipt of lysate with 10 micrograms of haIgG#1, i.p.. Groups (d) and (e) received 50 micrograms of AOA, i.p.. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies,except for groups (d) and (e) which were assayed for IgG1 anti-OA antibodies.

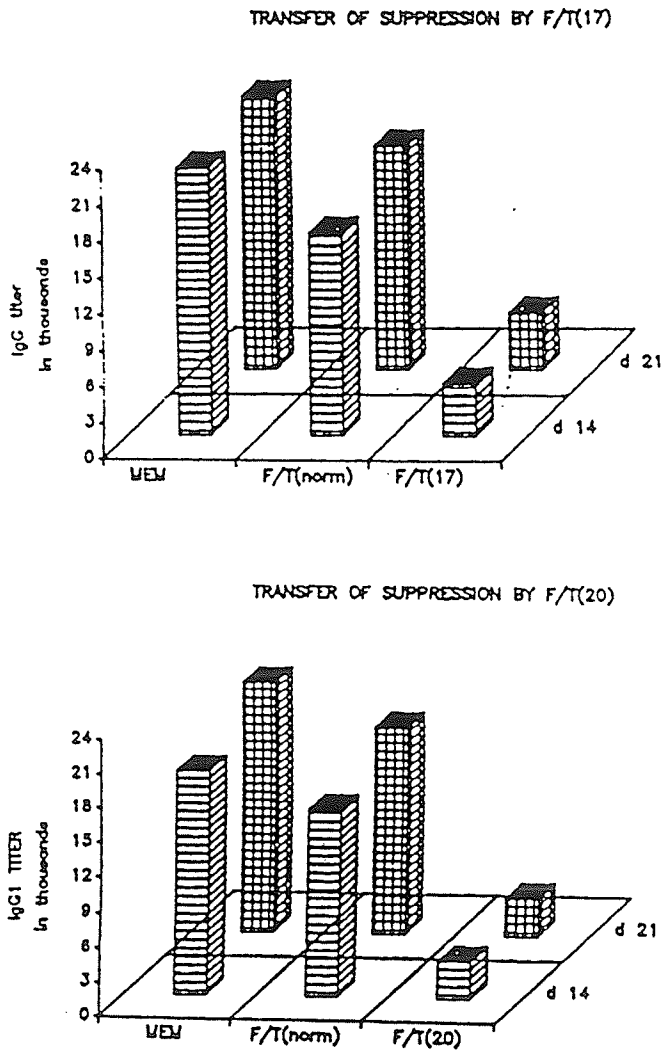
Group	Number of data points per mean
(a)	6
(b)	4
(c) to (e)	6
(f) to (h)	2

Figure 16; Legend
Transfer of suppression by F/T_{17} (upper)
Transfer of suppression by F/T_{20} (lower)

Upper figure
F/T(17) = groups of donor mice received 100 micrograms of HIgG(mPEG)17, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract of these cells was transferred, i.v., into naive syngeneic recipients.
F/T(norm) = groups of donor mice received PBS only, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations as described above. A freeze/thaw extract of the sIg- cells obtained was transferred, i.v., into naive syngeneic recipients.
MEM = groups of recipient mice received MEM only.
All recipient groups were immunised, i.p. with 50 micrograms of haIgG#2 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies.

Lower figure
F/T(20) = groups of donor mice received 100 micrograms of HIgG(mPEG)20, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract of these cells was transferred, i.v., into naive syngeneic recipients.
F/T(norm) = groups of donor mice received PBS only, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations as described above. A freeze/thaw extract of the sIg- cells obtained was transferred, i.v., into naive syngeneic recipients.
MEM = groups of recipient mice received MEM only.
All recipient groups were immunised, i.p. with 10 micrograms of haIgG#1 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Figure 16: TRANSFER OF SUPPRESSION BY F/T_{17} AND F/T_{20}



of MEM but approximately 5 fold greater than the average day 14 titer for the recipients of F/T_{20}.

Recipients of F/T_{20} that had been obtained from tolerant splenic leukocytes and treated with anti-Lyt-1.2 plus complement, anti-Lyt-2.2 plus complement or complement alone, showed average day 14 titers of 7,719, 13,879 and 6,548 respectively, compared with 16,058 for recipients of F/T_{normal} 3,222 for recipients of F/T_{20}.

Recipients of F/T_{20} that were immunised with AOA in place of haIgG showed no suppression (average day 14 titer of 11,902) compared with recipients of MEM which had also been immunised with AOA (average day 14 titer of 11,182). Similar titers were observed for day 21 bleeding.

Discussion

F/T_{20} extract is capable of transferring specific suppression into naive syngeneic recipients. F/T_{20} derived from tolerised leukocytes depleted of Lyt-2.2+ cells showed a markedly reduced ability to transfer tolerance whereas extract derived from Lyt-1.2 depleted cells did not; thus much of the suppressive ability of F/T_{20} is derived from Lyt-2.2 positive cells.

Jones and Kaplan (134) have shown that a soluble HGG specific factor obtained from the spleen cells of tolerant donors is able to mediate suppression. Tolerance to HGG was induced by the i.p. injection of 0.5 ml of 5 mg/ml ultracentrifuged HGG. The soluble factor was obtained from a suspension of whole spleen cells (i.e., no

fractionation of cells was performed) by sonication for 2 min on ice. The lysate was then centrifuged at 40,000 x g for 45 min and 0.2 ml of supernatant (at 5 x 10^7 cell equivalents/ml) used for injection into naive syngeneic recipients. Jones and Kaplan (134) were able to achieve >99% suppression with this factor whereas the F/T_{20} extract described here produced an average day 14 suppression of 80% (taking the titer for F/T_{normal} recipients as 100 %). There are, however, important differences between the two systems: (a) Jones and Kaplans' factor was induced by a large dose (2.5 mg) of de-aggregated HGG whereas the F/T_{20} extract was induced by 0.1 mg of mPEG conjugated HIgG; (b) Jones and Kaplans' factor was produced by sonication of a suspension of tolerant whole spleen cells whereas the F/T_{20} extract was produced by freezing and thawing the sIg- subpopulation of tolerant splenic leukocytes. Taniguchi and Miller (135) reported a similar HGG specific factor to that of Jones and Kaplan produced by a similar method.

Estimation of the molecular weight of the suppressive factor(s) present in F/T_{20} extracts and comparison with F/T_{normal} extract

In order to clarify whether or not the suppressive moiety present in the F/T_{20} extract did indeed have a molecular weight of less than 100 kD, the F/T20 extract was passed through a previously calibrated gel filtration column and the in vivo suppressive abilities of the fractions obtained determined. For purposes of comparison F/T_{normal} was also fractionated on the same column.

Materials and Methods

F/T_{20} and F/T_{normal} extracts were produced as described previously and applied, separately, at a concentration of 8 x 10^7 cell equivalents per ml to an AcA-44 gel filtration column (molecular weight fractionation range of 10-130 kD) equilibrated in PBS and 3.1 ml aliquots collected. This column had previously been calibrated for molecular weight determination using the following protein markers: blue dextran (c.2,000 kD), BSA (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD) and ribonuclease (13.7 kD). Following passage of the F/T_{20} extract through the column, aliquots were taken from the tube of highest O.D. within each peak, diluted as described below to a volume of 0.5 mls with PBS and injected i.v. into naive syngeneic recipients. The amount injected was normalised with respect to the amount of protein present on the lowest peak. Thus the O.D. of the lowest peak was considered to be 100 %. Peaks A, B, 1, 4, 7 and 10 were also injected without dilution into other recipients. An additional group received 0.25 ml of peak 1 and 0.25 ml of peak 4.

Recipients were immunised the following day and bled 14 and 21 days post-immunisation.

Results

The profile obtained following passage of the F/T_{20} extract through an ACA-44 column is presented in figure 17 and the IgG1 titers obtained for the in vivo testing of the eluate are presented in table 19.

Figure 17: Legend
Gel filtration profile of F/T_{20} after passage through AcA 44 column

Groups of mice received 100 micrograms of HIgG(mPEG)$_{20}$, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract of these cells was then passed through an ACA 44 gel filtration column. The resulting profile is presented here.

This column had been previously calibrated using the following markers:
67 = molecular weight calibration marker corresponding to Bovine serum albumin (67 kD).
43 = molecular weight calibration marker corresponding to ovalbumin (43 kD).
25 = molecular weight calibration marker corresponding to chymotrypsinogen A (25 kD).
13.7 = molecular weight calibration marker corresponding to ribouclease (13.7 kD).

Letters A, B and numbers 1 - 10 refer to fractions selected for in vivo testing for tolerogenic capacity (see table 19 and text).

Figure 17: GEL FILTRATION PROFILE OF F/T20 AFTER PASSAGE THROUGH AcA 44 COLUMN

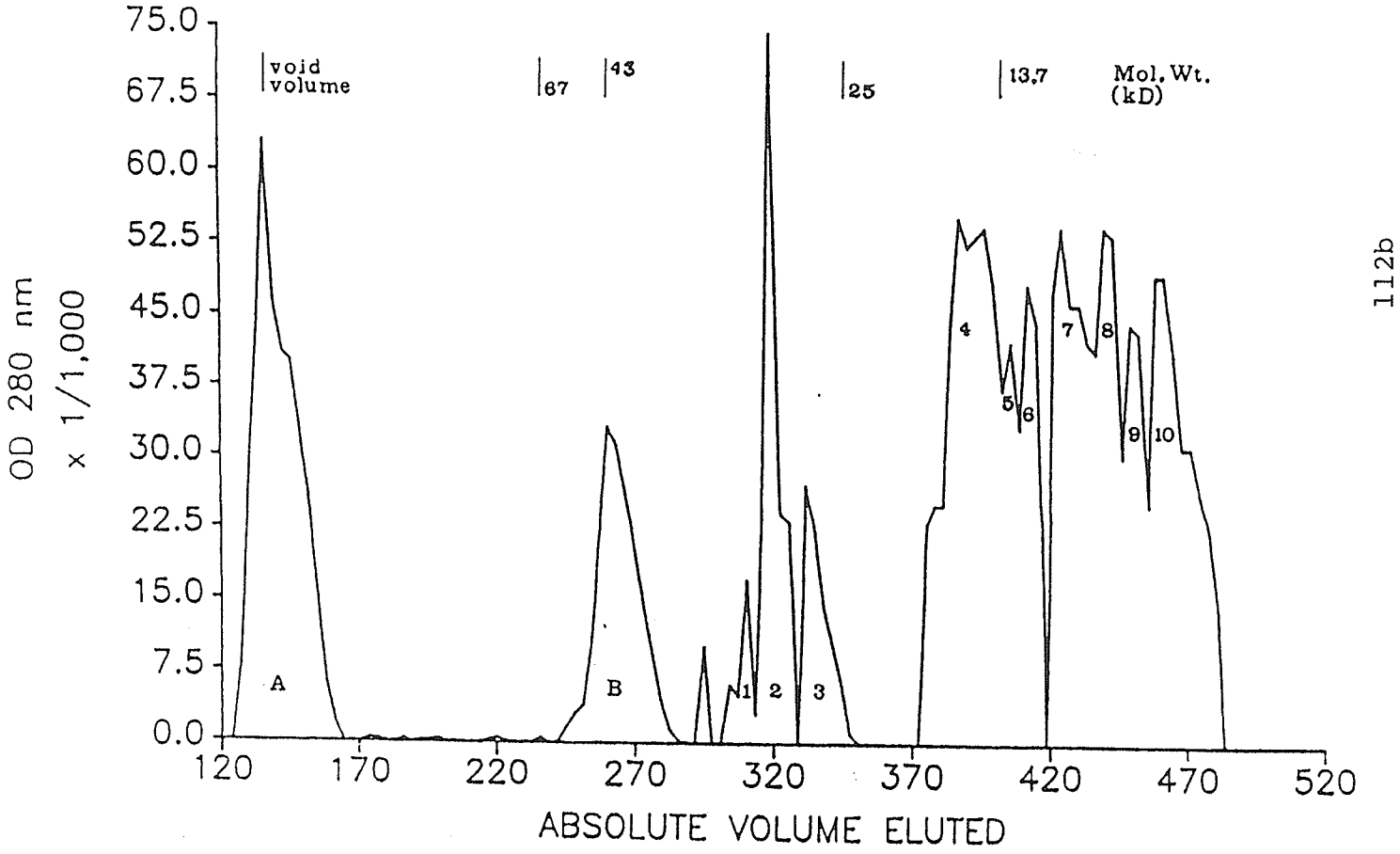


Table 19:
In vivo testing of F/T(20) extract fractions obtained after passage through AcA 44 column

Mean IgG1 anti-HIgG titer (2 data points per mean)

fraction	day 14	S.D.	percent supp'n	day 21	S.D.	percent supp'n
1	22,408	979	-14	19,857	424	8
2	11,885	122	40	13,283	369	38
3	20,997	1,200	-7	22,575	443	-5
4	3,895	155	80	5,111	163	76
5	3,747	155	81	4,583	97	79
6	2,908	1,282	85	4,030	263	82
7	7,138	501	64	8,824	228	59
8	23,045	2,189	-17	20,822	1,099	1
9	15,575	1,424	21	17,598	332	15
10	7,424	40	63	9,300	926	57
1	17,104	1,551	13	19,579	1,350	9
4	2,344	485	88	4,258	373	81
7	4,451	-	77	-	-	-
10	6,789	1,602	66	5,623	1,316	74
1+4	3,574	780	82	4,772	1,088	78
MEM	19,704	1,057	-	21,569	842	-
A	22,867	1,362	-16	22,539	2,507	-4
B	21,662	961	-9	22,791	2,031	-6

Splenic leukocytes were taken from donor mice that had been tolerised with 100 micrograms of HIgG(mPEG)20 six days earlier and were fraction -ated into sIg- cells by incubation on petri dishes coated with sheep anti-mouse Ig. A freeze/thaw extract was prepared from these cells and then passed through an AcA 44 column. Following passage through the column, fractions were collected corresponding to the highest OD of each peak, diluted to a total volume of 0.5 mls with PBS and inject -ed i.v. into naive syngeneic recipients. The amount injected was normalized with respect to the amount of protein in the smallest peak.

Other groups received undiluted material from peaks A, B, 1, 4, 7 and 10. An additional group received a mixture of 0.25 mls each of peaks 1 and 4. A control group received MEM only.

All groups were immunised one day after injection of fractions with 10 micrograms of haIgG#1, i.p.. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG1 anti-HIgG antibodies

Peaks A and B showed no suppressive ability when injected into naive syngeneic recipients. Suppressive ability was associated with some of the fractions eluting at > 260 ml i.e., fractions 2 and 4-7, 9 and 10. Combining fractions 1 and 4 (0.25 ml of each) resulted in suppression. This was done to see if there was any effect on combination of fractions.

The profile obtained following passage of the F/T_{normal} extract through an ACA-44 column is presented in figure 18 and the IgG1 titers obtained for the in vivo testing of the eluate are presented in table 20. No statistical difference was seen in fractions of F/Tnormal extract in comparison with one another and with recipients of MEM.

Discussion

The fractionation data presented here suggest that the suppressive factor(s) present in the F/T_{20} extract have a molecular weight in the region of 13.7-25 kD. No finer distinction can be made from the data obtained. It is possible that more than one suppressive factor is present in the extract, and that these have different molecular weights. Passage of the suppressive factor(s) through the column may dilute them and/or reduce their effectiveness. The F/T extract may contain proteases that are released from the lysed cells at the same time as the suppressive factor(s). These may reduce the effectiveness of the suppressive factor(s).

The HGG specific suppressive factor described by Jones and Kaplan

Figure 18: Legend
Comparison of suppressive regions of gel filtration profiles of F/T_{20}, F/T_{17} and F/T_{normal}

Groups of mice received 100 micrograms of $HIgG(mPEG)_{20}$, or 100 micrograms of $HIgG(mPEG)_{17}$ or PBS, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract of these cells was then passed through an ACA 44 gel filtration column. The resulting profiles are presented here.

This column had been previously calibrated using the following markers:
67 = molecular weight calibration marker corresponding to Bovine serum albumin (67 kD). Not shown here.
43 = molecular weight calibration marker corresponding to ovalbumin (43 kD). Not shown here.
25 = molecular weight calibration marker corresponding to chymotrypsinogen A (25 kD).
13.7 = molecular weight calibration marker corresponding to ribouclease (13.7 kD).

The regions shown here were found to contain fractions capable of inducing suppression when transferred, i.v., into naive syngeneic recipients (see tables 19, 20 and 23 and text).

These profiles are presented together to facilitate ease of comparison.

Figure 18: COMPARISON OF SUPPRESSIVE REGIONS OF GEL FILTRATION PROFILES OF F/T_{20}, F/T_{17} AND F/T_{normal}

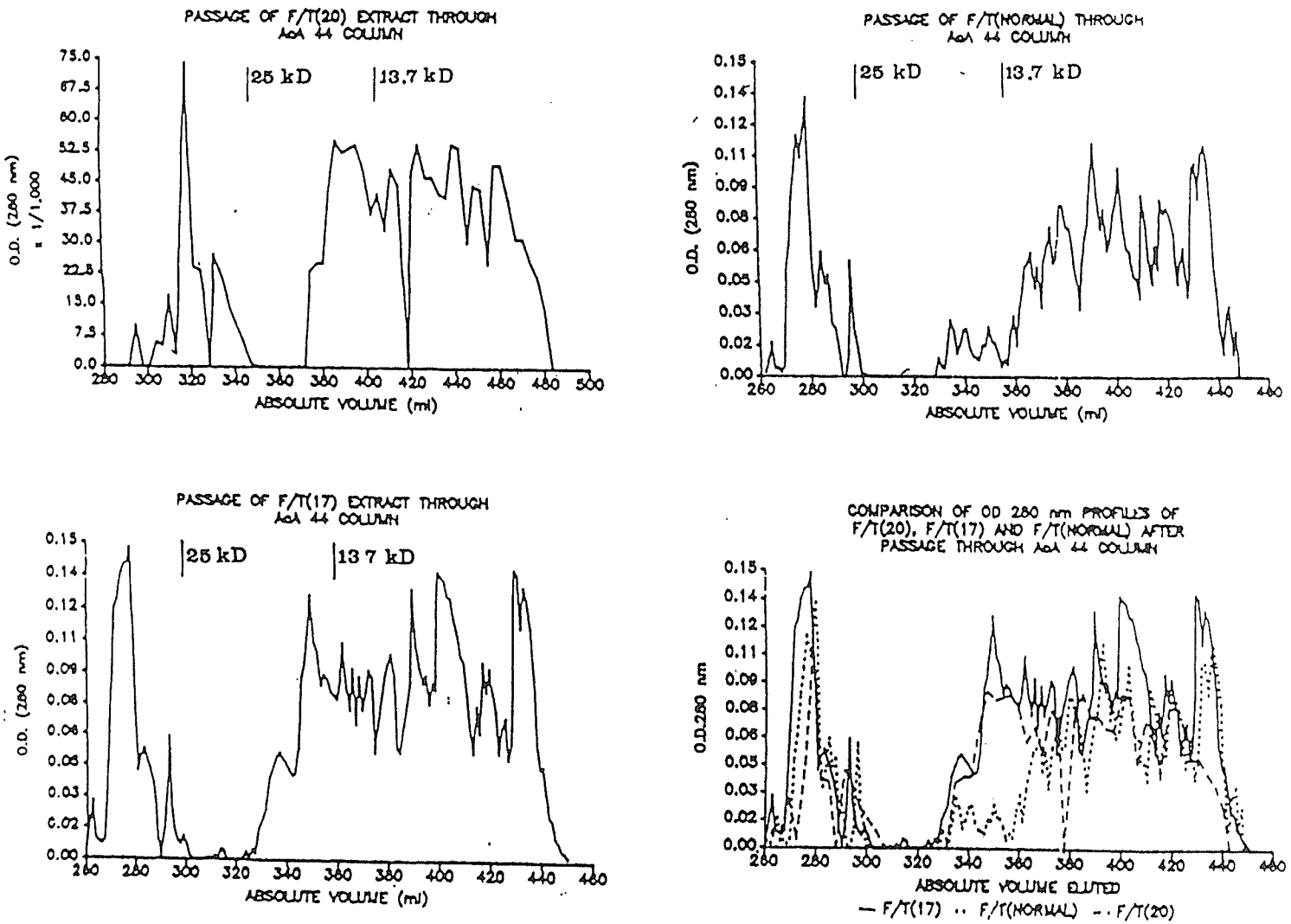


Table 20:
In vivo testing of F/Tnormal extract fractions obtained after passage through AcA 44 column

Mean IgG1 anti-HIgG titer (2 data points per mean)

fraction	day 14	S.D.	percent supp'n	day 21	S.D	percent supp'n
1	23,353	1,709	8	23,949	1,322	-16
2	20,278	1,023	20	20,317	2,493	2
3	20,011	1,382	21	21,777	2,070	-5
4	18,943	1,019	25	17,983	1,369	13
5	20,524	2,245	19	23,060	4,156	-11
6	19,065	383	25	21,235	1,302	-3
7	22,002	-	13	23,565	-	-14
8	22,419	3,613	12	18,577	2,217	10
9	21,499	4,940	15	22,195	5,286	-7
10	20,682	2,365	18	24,109	626	-17
1 (0.5)	21,983	45	13	21,497	2,454	-4
4 (0.5)	23,232	331	8	21,836	4,002	-6
7 (0.5)	19,730	387	22	21,785	2,498	-5
10 (0.5)	23,443	4,707	8	22,771	3,785	-10
1+4 0.25	21,615	2,099	15	20,845	2,080	-1
MEM (0.5)	25,344	489	0	20,687	808	0
A (0.5)	21,355	926	16	22,267	1,164	-8
B (0.5)	20,903	1,884	18	22,047	2,731	-7

Splenice leukocytes were taken from donor mice given PBS only 6 days earlier and were fractionated into sIg- cells by incubation on petri dishes coated with sheep anti-mouse Ig.

A freeze/thaw extract was prepared from these cells and then passed through an AcA 44 column. Following passage through the column, fractions were collected corresponding to the highest OD of each peak, diluted to a total volume of 0.5 mls with PBS and inject -ed i.v. into naive syngeneic recipients. The amount injected was normalized with respect to the amount of protein in the smallest peak.

Other groups received undiluted material from peaks A, B, 1, 4, 7 and 10. An additional group received a mixture of 0.25 mls each of peaks 1 and 4. A control group received MEM only.

All groups were immunised one day after injection of fractions with 10 micrograms of haIgG#1, i.p.. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG1 anti-HIgG antibodies

(134) had an apparent molecular weight of 45 kD and the elution profile of the whole spleen cell suspension from which it was derived, had only 2 peaks (Sephadex G-200 column).

In contrast the HGG specific suppressive factor described by Taniguchi and Miller (135) had a molecular weight in the range 30-55 kD and the elution profile of the whole spleen suspension from which it was derived had 5 peaks. (Sephadex G-200 column). Although the two groups had used different strains of mice (A/J and CBA/H/Wehi respectively) they both used 2.5 mg of deaggregated HGG to induce tolerance in the donor mice and sonication to produce the extracts - and yet the profiles of the two extracts, obtained on the same type of column, are different.

The F/T extracts used here, by contrast, were produced by freezing and thawing three times sIg- leukocytes derived from donor BDF1 mice tolerised with relatively low doses of conjugate (100 micrograms). Thus a subpopulation of spleen cells was selected for, before lysis of the cells. It is possible that the freezing and thawing process is more detrimental to the integrity of suppressive factors than the sonication process used by Jones and Kaplan and by Taniguchi and Miller because of the longer total time (15 mins compared with 2-3 mins for sonication on ice) and the thawing at 37^0C i.e., any proteases that are released from the cells upon lysis at the first freezing will be free to act throughout the remaining two thawings possibly compromising the suppressive ability of the extract.

The effect of treatment of F/T_{20} extract with soluble enzymes

Introduction

The chemical nature of the F/T_{20} extract was investigated by exposing the extract to various soluble enzymes and observing the effect on suppressive potency in vivo.

Materials and Methods

F/T_{20} extract was obtained as described previously and 4 x 10^7/ml sIg- cell equivalents incubated with either protease (Streptomyces griseus) (250 micrograms/ml), RNAse (200 micrograms/ml), DNAse (200 micrograms/ml) or MEM for 1 hr at 37^0C. All enzymes were purchased from Sigma Chemical Company, St. Louis, Mo.. The conditions used were based on those described by Jones and Kaplan (134). The F/T_{20} extract was then injected (2 x 10^7 cell equivalents per mouse) i.v. into naive syngeneic recipients. Recipients were immunised the following day and bled 14 and 21 days post-immunisation. Control groups received F/T_{20} treated with MEM or MEM in place of extract.

Results

IgG1 titers are presented in table 21(A) and the day 14 means are plotted in figure 19. Treatment of F/T20 extract with either RNAse, DNAse or MEM resulted in equivalent degrees of suppression; however treatment of F/T20 extract with Protease resulted in loss of suppressive activity with titers similar to those in untreated control animals.

Recipients of MEM had an average day 14 titer of 16,307 compared

Table 21:
The effect of enzymatic degradation upon freeze/thaw (F/T) extract

IgG1 titer

A: Soluble enzymes		Mean IgG1 anti-HIgG titer (data points per mean)			
group	enzyme treatment	day 14	S.D.	day 21	S.D.
(a)	Protease	15,520	1,853	17,728	2,083
(b)*	RNAse	4,578	1,208	4,849	519
(c)*	DNAse	2,571	775	4,807	1,435
(d)	no F/T	16,307	1,282	20,076	4,057
(e)*	MEM	3,256	486	2,951	622

B: Insoluble enzyme		Mean IgG1 anti-HIgG titer (data points per mean)			
group	enzyme treatment	mean	S.D.	mean	S.D.
(a)*	MEM	5,339	796	4,558	453
(b)	Protease	16,572	904	15,001	611
(c)	no F/T	21,935	2,714	21,477	1,397

Part A:
Splenic leukocytes were taken from donor mice that had been tolerised with 100 micrograms of HIgG(mPEG)20 six days earlier and were fraction-ated into sIg- cells by incubation on petri dishes coated with sheep anti-mouse Ig. A freeze/thaw extract was prepared from these cells which was then incubated with protease (group (a)), RNAse (group (b)) DNAse (group(c)) or MEM only (group (e)). Group (d) received no extract. All enzymes were soluble, i.e., not bound to solid supports.

All groups were immunised one day after injection of fractions with 10 micrograms of haIgG#1, i.p.. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG1 anti-HIgG antibodies

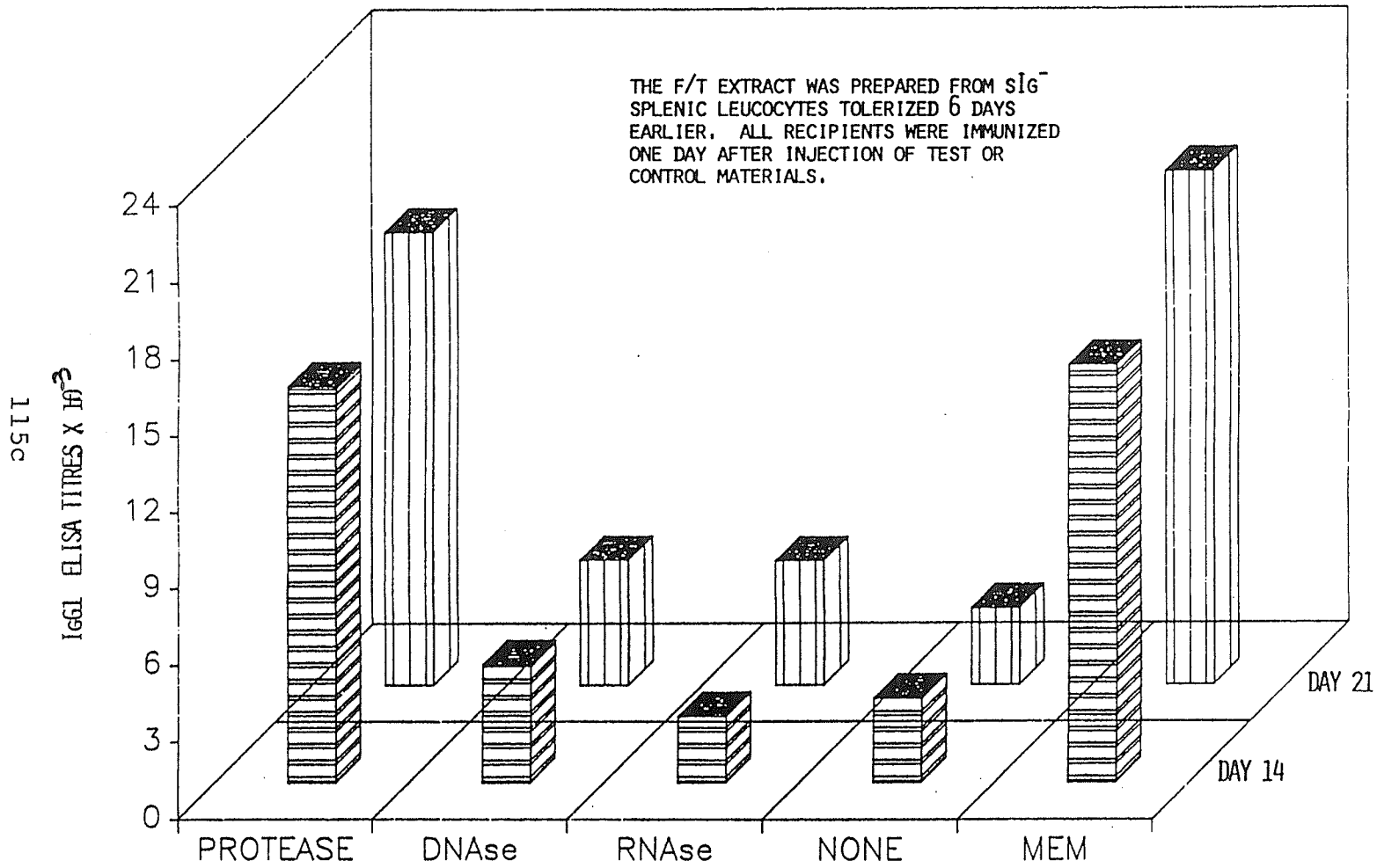
Part B:
The above experiment was repeated only this time insoluble enzymes were used in place of soluble.

Figure 19: Legend
Effect of enzyme treatment on tolerogenic activity of freeze/thaw (F/T) extract

Groups of mice received 100 micrograms of HIgG(mPEG)$_{20}$, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract of these cells was incubated with one of the following: protease, RNAse, DNAse or MEM and then transferred, i.v., into naive syngeneic recipients. A control group of recipients received no extract but was given MEM ("NONE").

All recipient groups were immunised, i.p., with 10 micrograms of haIgG#1 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG1 anti-HIgG antibodies.

Figure 19: EFFECT OF ENZYME TREATMENT ON TOLEROGENIC ACTIVITY OF FREEZE/THAW (F/T) EXTRACT



with 3,256 (80% suppression) for recipients of F/T_{20} that had been incubated with MEM. Recipients of F/T_{20} that had been incubated with either DNAse or RNAse had similar average day 14 titers (3,071 and 4,578 respectively) to that for recipients of F/T_{20} incubated with MEM (3,256) which is 81% and 72% suppression respectively. However recipients of F/T_{20} that had been incubated with protease had an average day 14 titer of 15,520 (5% suppression). Average titer values were similar for day 21 bleedings.

Discussion

The suppressive factor(s) present in F/T_{20} extract are at least in part made of protein. It is not possible to definitely exclude the presence of RNA or DNA on the basis of these experiments because although the RNAse and DNAse were freshly obtained there may not have been equivalent optimal conditions for all three enzymes, RNAse, DNAse and Protease. In addition these enzymes were soluble and were not removed before injection of the F/T_{20} extract into the recipient.

These experiments were based on the method of Jones and Kaplan (134) who reported 96% and 97% suppression in recipients of their suppressive factor after it had been incubated with DNAse and RNAse respectively and only 16% suppression in mice receiving protease treated factor.

Treatment of F/T_{20} with insoluble Protease (Streptomyces griseus) bound to agarose

Introduction

In the above experiments the enzymes used were soluble and were injected into the recipient mice together with the F/T_{20} extract. In order to avoid this complication insoluble Protease i.e., covalently bound to agarose (Sigma Chemical Company, St Louis, Mo.) was incubated with F/T_{20} in place of soluble Protease.

Materials and Methods

F/T_{20} was obtained as described previously and 4×10^7 /ml sIg-cell equivalents of F/T_{20} were incubated with 10 mg of insoluble Protease (Streptomyces griseus), which had been previously washed several times with double distilled water before use, for 1 hr at 37^0C with gentle shaking. The insoluble enzyme was then removed by centrifugation (2,000 rpm, 10 mins) and 2×10^7 cell equivalents injected, i.v., into naive syngeneic recipients. Control groups received either MEM or F/T_{20} that had been incubated with MEM. All recipients were immunised the following day and bled 14 and 21 days post-immunisation.

Results

IgG1 titers are presented in table 21(B). Treatment of F/T20 with insoluble Protease resulted in loss of suppressive activity with titers similar to those in untreated control animals. Treatment of the extract with MEM resulted in suppression i.e. MEM had no effect.

Recipients of F/T_{20} ~~that had been~~ incubated with MEM had an average day 14 titer of 5,339 compared with 21,935 for recipients of MEM in place of F/T_{20} which represents 76% suppression. However recipients of F/T_{20} that had been incubated with Protease had an average day 14 titer of 16,572 which represents 25% suppression.

Discussion

Reduction in the suppressive ability of F/T_{20} was observed if the extract is incubated with insoluble Protease although to a lesser extent than was observed for soluble Protease in the previous experiment viz, 25% and 5% suppression respectively compared with 76% and 80% respectively for recipients of F/T_{20} that had been incubated with MEM in place of Protease. The difference in the results obtained using the soluble and insoluble Proteases may reflect differences in specific activity and/or the effect of introducing soluble Protease into recipients.

PART II: The Transfer of suppression by F/T_{17} extract

Transfer of suppression by F/T_{17} extract of sIg- tolerised splenic leukocytes

Introduction

The transfer of suppression by tolerant sIg- leukocytes derived from donors tolerised with 100 micrograms of $HIgG(mPEG)_{17}$ was demonstrated previously. The ability of an F/T extract of these cells (F/T_{17}) to transfer suppression was examined and compared with data obtained for F/T_{20} and for F/T_{normal}.

In addition, F/T extracts derived from tolerised splenic leukocytes that had been treated with anti-Lyt monoclonal antibodies plus complement was examined for their tolerogenic properties.

Materials and Methods

F/T extracts were made as described previously; F/T_{17} being obtained from 2×10^7 sIg- leukocytes (or 5×10^7 unfractionated leukocytes in the case of the F/T_{20} obtained from cells treated with anti-Lyt-1.2 plus complement, anti-Lyt-2.2 plus complement or complement alone: see chapter 3) derived from donor mice given 100 micrograms of $HIgG(mPEG)_{17}$, i.v., 6 days prior to sacrifice and F/T_{normal} being obtained from the same number of sIg- leukocytes derived from donor mice given PBS, i.v., 6 days prior to sacrifice. The F/T extracts or MEM were injected, i.v., into naive syngeneic recipients which were immunised the following day and bled 14 and 31

days post-immunisation. Control groups were immunised with AOA in place of HaIgG.

Results

IgG titers are presented in table 22 and the means are plotted in figure 16, upper left quadrant (AOA titers not plotted). Mice treated with F/T17 extract showed suppression in comparison to mice receiving MEM. Mice receiving F/T_{normal} (i.e., extract derived from donor mice given PBS) showed some suppression also but not to the same degree as that induced by F/T17.

The average day 14 titer for recipients of F/T_{17} was 4,336 compared with 22,107 for recipients of MEM (80% suppression). Recipients of F/T_{normal} had average day 14 titers of 16,767 (24% suppression).

Recipients of F/T_{17} that were immunised with AOA in place of haIgG showed no suppression (average day 14 titer of 13,892) compared with recipients of MEM which had also been immunised with AOA (average day 14 titer of 14,360).

Recipients of extract derived from Lyt-1.2 depleted cells showed an average day 14 titer of 7,680 compared with 14,063 for extract derived from Lyt-2.2 depleted cells or 7,968 for extract derived from complement treated cells. Titers were similar for day 21 bleedings.

Discussion

F/T_{17} extract is capable of transferring specific suppression into naive syngeneic recipients. F/T_{17} derived from tolerised leukocytes

Table 22:
Transfer of tolerance by F/T(17)

donor	donor	recipi-ent	Mean IgG anti-HIgG titer (group (d) and (e) anti-OA)			
	d -6	d0	day 14	S.D.	mean	S.D.
(a)	-	MEM	22,107	1,260	22,593	2,080
(b)	PBS	F/Tn	16,767	1,637	18,467	843
(c)*	conj.	F/T17	4,336	881	4,676	312
(d)	-	MEM	14,360	1,217	13,709	1,932
(e)	conj	F/T17	13,892	1,778	13,332	1,305
(f)*	"	F/T17lyt1	7,680	932	10,663	466
(g)*	"	F/T17lyt2	14,063	1,329	16,409	2,153
(h)*	"	C	7,968	2,780	9,486	1,981

Groups of mice were injected with 100 micrograms of HIgG(mPEG)17 or PBS, i.v. and killed 6 days later. Their splenic leukoctytes were fractionated into sIg- cells by incubation on petri dishes coated sheep anti-mouse Ig. A freeze/thaw lysate of these cells was then injected i.v. into naive syngeneic recipients. Other mice received MEM only (groups (a) and (d). Group (f) received lysate derived from HIgG(mPEG)17 treated donors whose unfractionated splenic leukocytes had been incubated with anti-Lyt 1.2 + complement prior to injection into recipients. Group (g) received lysate derived from HIgG(mPEG)17 treated donors whose unfractionated splenic leukocytes had been incub-ated with anti-Lyt 2.2 plus complement. Group (h) received lysate derived from HIgG(mPEG)17 treated donors whose unfractionated splenic leukocytes had been treated with complement only.

Groups (a) to (c) and (f) to (h) were immunised one day following receipt of lysate with 50 micrograms of haIgG#2, i.p.. Groups (d) and (e) received 50 micrograms of AOA, i.p.. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies, except for groups (d) and (e) which were assayed for IgG anti-OA antibodies.

Group	Number of data points per mean
(a) to (e)	4
(f) to (h)	2

depleted of Lyt-2.2+ cells showed a markedly reduced ability to transfer tolerance whereas extract derived from Lyt-1.2 depleted cells did not; thus much of the suppressive ability of F/T_{20} is derived from Lyt-2.2 positive cells. [Comparison of F/T extract with other HGG specific factors was made previously under the section on F/T_{20} tolerance transfer.]

Estimation of the molecular weight of the suppressive factor(s) present in F/T_{17} extract and comparison with F/T_{normal} extract

Introduction

It has been shown previously that the amount of suppression induced by $HIgG(mPEG)_n$ conjugates is related to the degree of substitution n for a dose of 100 micrograms per mouse. Therefore it was of interest to discover whether or not the gel filtration profile and molecular weight estimate obtained for F/T_{17} would be similar to those obtained for F/T_{20}. The F/T_{17} extract was therefore passed through a previously calibrated gel filtration column and the in vivo suppressive abilities of the fractions thereby obtained were determined. [Note:this was not the same AcA 44 column as used previously for the fractionation of F/T_{20} and F/T_{normal}. This was due to technical problems with the original column].

Materials and Methods

F/T_{17} extract was produced as described previously and applied, at a concentration of 8×10^7 cell equivalents per ml to an AcA-44 gel filtration column (molecular weight fractionation range of 10-130 kD)

equilibrated in PBS and 1.25 ml fractions collected. Recipient mice were injected i.v. with 0.5 mls taken from the peak tubes of the fractions collected as opposed to attempting to normalise as was done for F/T_{20}; because it was possible that the OD profile obtained may not necessarily reflect suppressive material per se but could be some other material that co-fractionates with suppressive material. Any attempt at normalising concentrations would in this instance be questionable as one may dilute out the suppressive material if it happens to co-fractionate with other material giving a relatively high OD..

This column had previously been calibrated for molecular weight determination using the following protein markers: blue dextran (c.2,000 kD), BSA (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD) and ribonuclease (13.7 kD). Recipients were immunised the following day and bled 14 and 21 days post-immunisation.

Results

The profile obtained following fractionation of the F/T_{17} extract was similar to that obtained for the F/T_{20} extract with peaks corresponding to peaks A and B in figure 18. 0.5 ml aliquots of these peaks showed no suppressive ability when injected into naive syngeneic recipients. Suppressive ability was associated with some of the fractions eluting at > 260 ml i.e., fractions 4-7,9 and 10. Combining fractions 1 and 4 (0.25 mls of each) resulted in suppression. This was done to see if there was any effect on

combination of fractions. This section of the profiles is presented in figure 18 lower left quadrant. IgG titers obtained for the in vivo testing of the fractions are presented in table 23.

Following the method adopted for the in vivo suppression data for F/T_{20} fractionation, if the percent suppression associated with each fraction from the F/T_{normal} profile is compared with the corresponding percent suppression observed for each fraction of the F/T_{17} profile then it is apparent that the suppressive factor(s) present in F/T_{17} extract lie in the the elution range of 260 - 480 ml.

Discussion

The fractionation data presented here suggest that the suppressive factor(s) present in the F/T_{17} extract have a molecular weight in the same range as that determined for F/T_{20} extract i.e., 13.7-25 kD.

[Comparison with the findings of other workers was made previously under the Discussion section of the section describing the fractionation of F/T_{20} on an AcA 44 column].

Characterisation of some antigenic determinants present on F/T_{17}

Introduction

It has been shown previously that the suppression transferred by F/T_{17} is antigen specific. Therefore the suppressive factor(s) present in F/T_{17} must carry some antigenic information. This was investigated by adsorbing the F/T_{17} extract with various immunoglobulins and anti-sera to these immunoglobulins.

Table 23:
In vivo testing of F/T(17) extract fractions obtained after passage through AcA 44 column

Mean IgG anti-HIgG titer (2 data points per mean)

fraction	day 14	S.D.	percent supp'n	day 21	S.D.	percent supp'n
1	24,533	1,243	-3	20,580	658	18
2	13,551	-	43	14,582	-	42
3	26,003	2,816	-10	24,880	1,080	1
4*	9,309	907	61	9,860	232	61
5*	7,307	503	69	8,768	320	65
6*	6,722	1,727	72	6,383	88	74
7*	11,610	1,924	51	11,020	1,364	56
8	20,755	1,048	12	24,823	236	1
9*	17,989	13	24	19,388	235	23
10*	10,305	1,349	57	12,405	1,358	50
1	22,810	3,739	4	23,059	4,157	8
4*	6,185	1,660	74	7,567	801	70
7	11,573	1,049	51	11,449	7	54
10	14,745	1,003	38	16,878	608	33
1+4	7,878	194	67	8,572	615	66
MEM	23,711	1,666	-	25,027	668	-
A	27,750	1,089	-17	27,356	1,336	-9
B	23,662	1,911	-	26,706	1,006	-7

Splenic leukocytes were taken from donor mice that had been tolerised with 100 micrograms of HIgG(mPEG)17 six days earlier and were fraction -ated into sIg- cells by incubation on petri dishes coated with sheep anti-mouse Ig. A freeze/thaw extract was prepared from these cells and then passed through an AcA 44 column. Following passage through the column, fractions were collected corresponding to the highest OD of each peak, diluted to a total volume of 0.5 mls with PBS and inject -ed i.v. into naive syngeneic recipients. The amount injected was normalized with respect to the amount of protein in the smallest peak.

Other groups received undiluted material from peaks A, B, 1, 4, 7 and 10. An additional group received a mixture of 0.25 mls each of peaks 1 and 4. A control group received MEM only.

All groups were immunised one day after injection of fractions with 50 micrograms of haIgG#2, i.p.. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG anti-HIgG antibodies.

Materials and Method

F/T_{17} extract at a concentration of 8×10^7 sIg- cell equivalents per ml, was incubated with the following proteins all at a concentration of 1.24×10^{-6} moles, overnight, at 4^0C with shaking: mouse anti-bovine IgG (maBIgG), mouse anti-human IgG (maHIgG), human IgG myeloma used to make conjugate $HIgG(mPEG)_{20}$ (HIgG#1), bovine IgG (BIgG), ovalbumin (OA), human IgG used to make conjugate $HIgG(mPEG)_{17}$ (HIgG#2), and MEM. Each extract/protein mixture was then placed in an Amicon chamber fitted with an XM50 ultrafiltration membrane. The chamber was run to dryness at a pressure of 30 psi and the filtrate injected into naive syngeneic recipients.

Recipient mice were immunised the following day and bled 14 and 21 days post-immunisation.

Results

IgG titers are presented in table 24 and means are plotted in figure 20.

Incubation of F/Tl7 with maHIgG#2, maHIgG#1 or maBIgG had no effect on its suppressive capacity. However, incubation with HIgG#2, HIgG#1 or BIgG all reduced its suppressive capacity (to the same degree) but not to levels equivalent to PBS controls i.e., some suppressive capacity still remained.

Recipients of F/T_{17} that had been incubated with PBS had an average day 14 titer of 4,323 compared with 21,867 for recipients of MEM in place of F/T_{17} i.e., 80% suppression. Incubation of F/T_{17}

Table 24:
Characterisation of some antigenic determinants present on F/T(17)

group	d -1		Mean IgG anti-HIgG titer (2 data points per mean) day 14	S.D.	day 21	S.D.
(a)*	HIgG#2	+ F/T	16,681	894	17,213	1,086
(b)*	maHIgG#2	"	4,569	1,884	4,339	308
(c)*	HIgG#1	"	14,267	1,779	16,280	2,427
(d)*	maHIgG#1	"	4,084	1,462	5,704	1,143
(e)*	BIgG	"	14,214	1,292	14,776	1,727
(f)*	maBIgG	"	4,941	1,231	4,739	1,029
(g)*	PBS	"	4,323	301	4,642	489
(h)	"	MEM	21,867	1,744	23,831	2,357

Splenic leukocytes were taken from donor mice that had been tolerised with 100 micrograms of HIgG(mPEG)17 six days earlier and were fraction -ated into sIg- cells by incubation on petri dishes coated with sheep anti-mouse Ig. A freeze/thaw extract was prepared from these cells and injected, i.v., into naive syngeneic recipients after being first incubated with one of the following :
group (a) human IgG#2
group (b) mouse anti-Human IgG#2
group (c) human IgG#1
group (d) mouse anti-human IgG#1
group (e) bovine IgG
group (f) mouse anti-bovine IgG
group (g) PBS
group (c) PBS
All groups were immunised one day after injection of fractions with 50 micrograms of haIgG#2, i.p.. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG anti-HIgG antibodies.

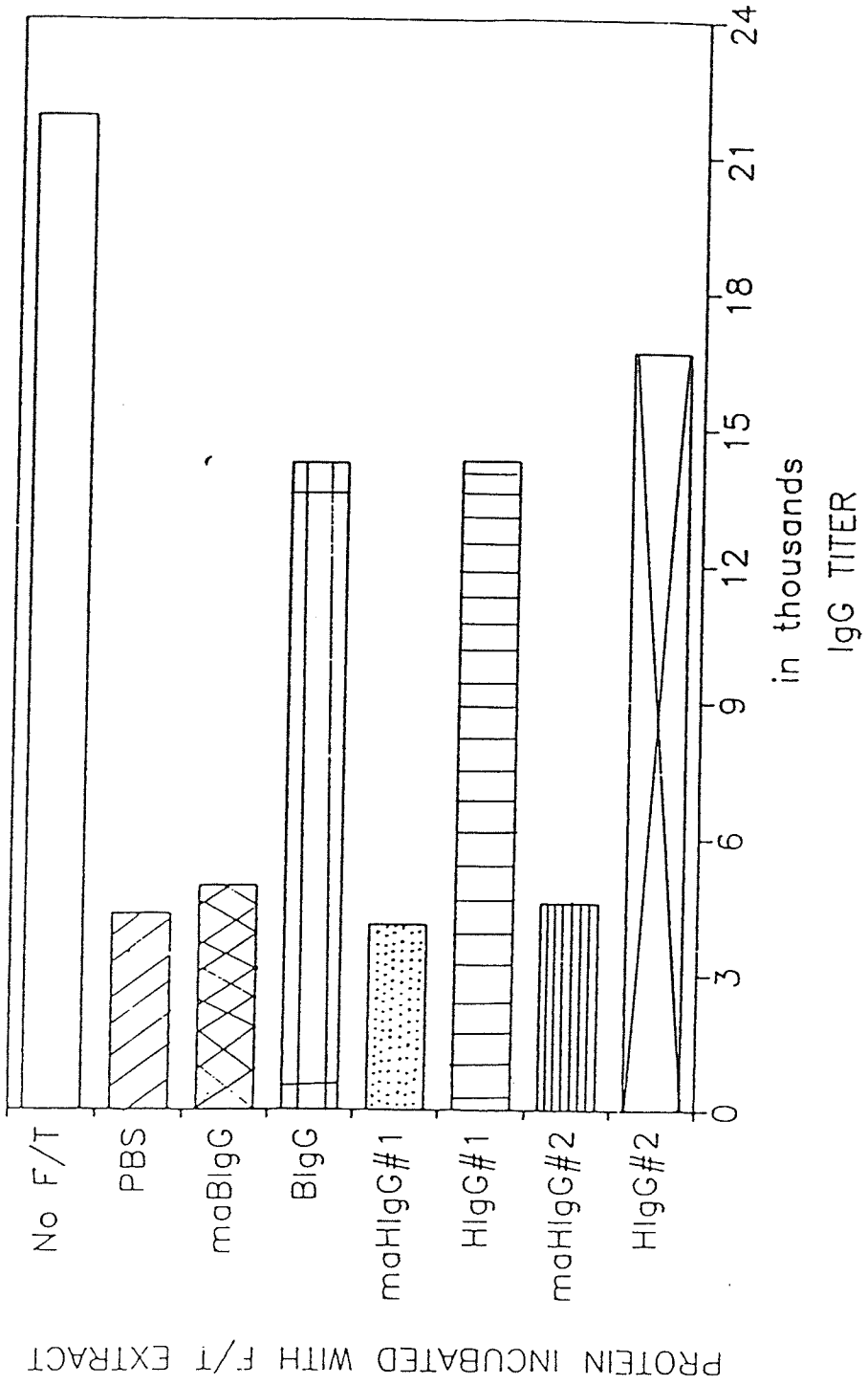
Figure 20: Legend
Characterisation of some antigenic determinants present on F/T(17)

Groups of mice received 100 micrograms of HIgG(mPEG)$_{17}$, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract of these cells was incubated with one of the following:

maBIgG = mouse anti-bovine IgG antiserum
BIgG = Bovine IgG
maHIgG#1 = mouse anti-human HIgG#1 antiserum
maHIgG#2 = mouse anti-human HIgG#2 antiserum
HIgG#1 = human IgG myeloma #1
HIgG#2 = human IgG myeloma #2
PBS = phosphate buffered saline

Following incubation the above mixtures were then injected, i.v., into naive syngeneic recipients. A control group of recipients received MEM only. All recipient groups were immunised, i.p., with 50 micrograms of haIgG#2 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies.

Figure 20: CHARACTERISATION OF SOME ANTIGENIC DETERMINANTS PRESENT ON F/T(17)



with anti-HIgG#1, anti-HIgG#2 or with anti-BIgG had little effect on the suppressive ability of the extract with average day 14 titers of 4,084, 4,564 and 4,941 respectively. However, incubation of F/T_{17} with HIgG#1, HIgG#2 or with BIgG did effect the suppressive ability of the extract with average day 14 titers of 14,267, 16,681 and 14,214 respectively. Titers were similar for day 21 bleedings.

Discussion

The suppressive ability of F/T_{17} can be reduced by incubation of the extract with BIgG and with two HIgG myelomas. It appears, therefore, that the suppressive factor(s) present in F/T_{17} extract recognise some common determinant(s) present on the IgG molecules.

The suppressive ability of F/T_{17} cannot be reduced by incubation of the extract with antisera to the above IgG molecules under the stated conditions; implying that the HIgG from the conjugate was not associated with the suppressive factor(s) present in the F/T_{17} extract. This is supported by the estimated molecular weight range of 13.7-25 kD for the suppressive factor(s) compared with 160 kD for intact HIgG. However, despite the fact that the antisera were all produced by hyperimmunisation protocols involving Freund's adjuvant, there remains the possibility that the anti-IgG antibodies in the antisera were not present in sufficient quantities and/or of high enough affinities to adsorb out the suppressive factor(s) in the F/T_{17} extract.

Taniguchi and Miller (135) were able to adsorb out their HGG

specific suppressor factor by passage of the factor through an HGG immunoadsorbent column but not by its passage through columns of anti-HGG. Similar findings were reported by Jones and Kaplan (134) for their HGG specific suppressor factor in that its suppressive ability could be reduced after passage through an HGG-Sepharose 4B column but not by passage through a rabbit anti HGG-Sepharose 4B column.

Genetic restriction on the transfer of suppression by F/T_{17} extract

Introduction

It has been shown previously that the suppressive factor(s) present in the F/T_{17} extract is antigen specific and that the factor(s) can be adsorbed with HIgG viz, the factor carries information about the nature of the antigen used to induce it. Determining whether or not the F/T_{17} extract could transfer suppression across the major histocompatibility complex (MHC) barrier would give some insight into what additional information the suppressive factor(s) carry i.e., whether or not it carried information pertaining to the MHC. This was investigated by studying the suppressive ability of F/T_{17} extract derived from BDF1 mice, when injected into naive syngeneic recipients of the following strains: C57BL/6, DBA/2 (i.e., the parental strains of BDF1 mice) or C3H (i.e., unrelated haplotype to the other three strains).

Materials and Method

F/T_{17} was obtained as described previously from BDF1 donor mice

and 2 x 10^7 sIg- cell equivalents injected, i.v. into naive syngeneic recipients of the following strains of mice: BDF1 (haplotype: b/d), C57BL/6 haplotype: b), DBA/2 (haplotype: d) or C3H (haplotype: k). Control groups received MEM in place of F/T_{17}. Other control groups were given 100 micrograms of $HIgG(mPEG)_{17}$ 6 days prior to immunisation in order to be sure that any lack of an effect observed in strains receiving the F/T_{17} were not simply due to an innate inability of that strain to be tolerised by conjugate in the first place. All F/T_{17} recipients were immunised the following day and all groups were bled 14 and 21 days post-immunisation.

Results

IgG titers for recipients of F/T_{17} and MEM are presented in table 25(A) and the average percent suppressions are plotted in figure 21. IgG titers for control groups receiving conjugate are presented in table 25(B).

Considering, table 25(A): F/T17 extract derived from BDF1 mice induced suppression when administered to BDF1 mice (haplotype b/d), C57BL/6 mice (haplotype b) and to DBA/2 mice (haplotype d) but not in C3H mice (haplotype k). The degree of suppression induced in the first three above mentioned strains wass equivalent.

Injection of $HIgG(mPEG)_{17}$ per se into any of the above mentioned strains resulted in equally profound suppression (table 25 (B)).

Considering, table 25(A) BDF1 mice and both parental strains could be suppressed by F/T_{17} extract showing 80%, 79% and 68% suppression

Table 25:
H-2 restriction to the transfer of tolerance by F/T(17)

(A)

group	strain	d 0	Mean IgG anti-HIgG titer (2 data points per mean) day 14	S.D.	day 21	S.D.
(a)*	BDF1	F/T	4,350	1,496	4,591	218
(b)	"	MEM	22,348	1,219	20,608	1,131
(c)*	C57BL/6	F/T	4,569	624	5,051	315
(d)	"	MEM	22,315	1,544	22,892	1,647
(e)*	DBA/2	F/T	5,754	930	4,724	204
(f)	"	MEM	17,760	639	17,845	2,759
(g)	C3H	F/T	16,105	1,276	16,794	1,959
(h)	"	MEM	15,145	1,227	16,500	1,221

(B)

	strain	d -6	Mean IgG anti-HIgG titer (2 data points per mean) day 14	S.D.	day 21	S.D.
(i)*	BDF1	conj.	4,210	1,703	5,655	969
(j)	"	PBS	21,886	2,667	23,976	2,775
(k)*	C57BL/6	conj.	6,255	1,619	7,843	533
(l)	"	PBS	19,708	572	23,189	1,314
(m)*	DBA/2	conj.	7,255	1,521	7,861	496
(n)	"	PBS	17,744	1,571	18,806	857
(o)*	C3H	conj.	7,611	1,258	8,996	246
(p)	"	PBS	16,971	1,061	18,662	829

Part A:
Splenic leukocytes were taken from donor mice that had been tolerised with 100 micrograms of HIgG(mPEG)17 six days earlier and were fraction-ated into sIg- cells by incubation on petri dishes coated with sheep anti-mouse Ig. A freeze/thaw extract was prepared from these cells and injected into naive recipients of the following strains of mice: BDF1 (haplotype: b/d = group (a)), C57BL/6 (haplotype: b = group (c)), DBA/2 (haplotype: d = group (e) and or C3H (haplotype: k = group (g). Control groups received MEM in place of extract (groups (b), (d), (f) and (h)).
All groups were immunised one day after injection of fractions with 50 micrograms of haIgG#2, i.p.. All groups were bled 14 and 21 days post immunisation and their sera assayed for IgG anti-HIgG antibodies.

Part B:
Other groups of mice received 100 micrograms of HIgG(mPEG)17 or PBS i.v. and were immunised and bled as above (Part A). These groups were included in order to test whether or not the strains included in Part A were susceptible to tolerisation by HIgG(mPEG)17 per se.

Figure 21: Legend
H-2 restriction to the transfer of tolerance by F/T(17)

Groups of mice received 100 micrograms of $HIgG(mPEG)_{17}$, i.v.. Six days later these mice were killed and their splenic leukocytes fractionated into sIg- and sIg+ populations by incubation on petri dishes coated with sheep anti-mouse Ig. Cells which did not adhere to these plates = sIg-. A freeze/thaw extract (F/T_{17}) of these cells was then injected into the following strains of mice:

BDF1: haplotype = b/d
C57BL/6: haplotype = b
DBA/2: haplotype = d
C3H: haplotype = k

Control recipient groups received MEM in place of extract. All recipient groups were immunised, i.p., with 50 micrograms of haIgG#2 the following day. All groups were bled 14 and 21 days post-immunisation and their sera assayed for IgG anti-HIgG antibodies.

Percent suppression, for a given strain = 100 - (mean IgG titer for recipient of F/T_{17}/ mean IgG titer for recipient of MEM x 100).

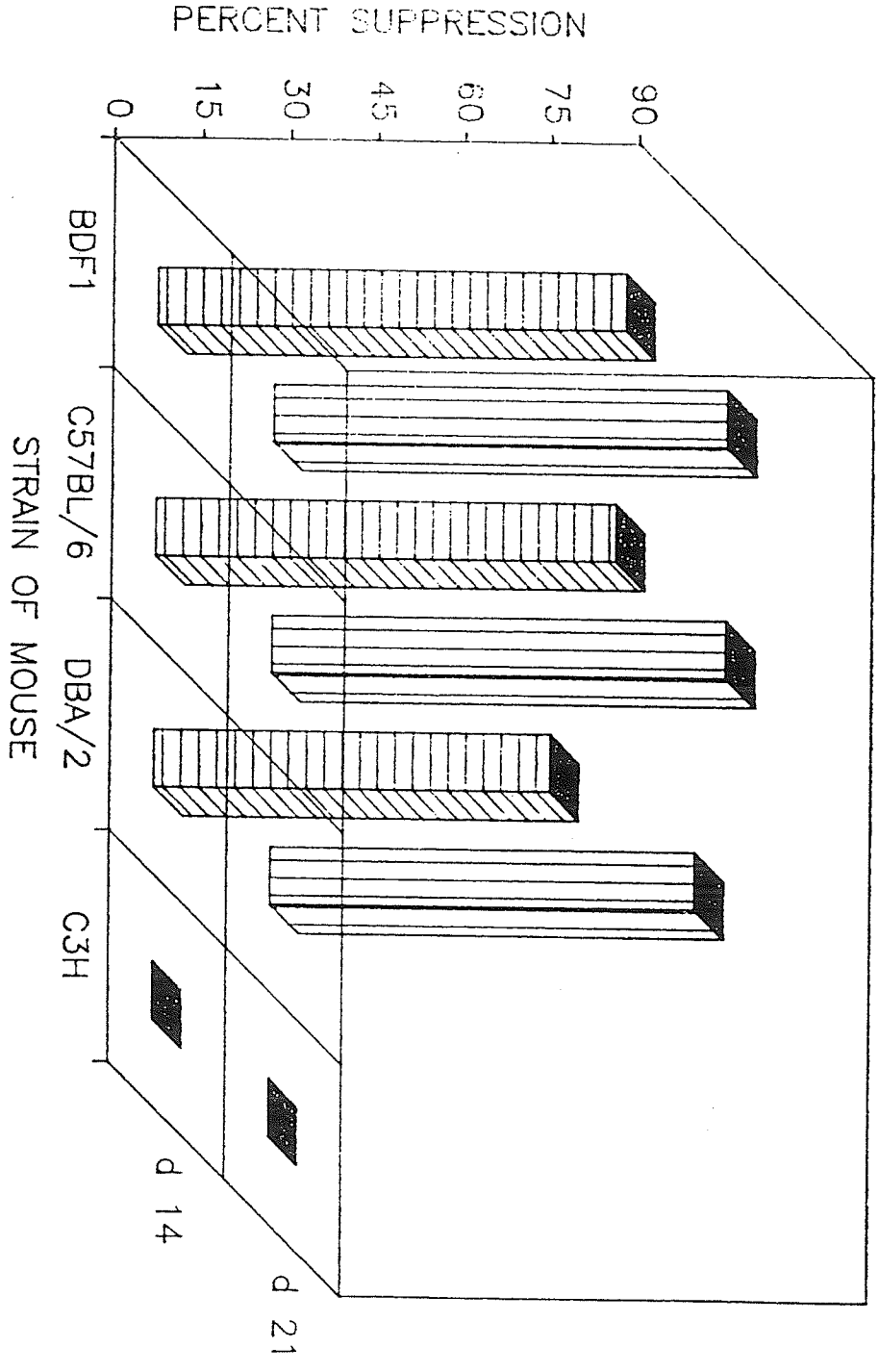


Figure 21: H-2 RESTRICTION TO THE TRANSFER OF TOLERANCE BY F/T(17)

respectively. [Percent suppression must be used for comparison, as opposed to titers per se, as a consequence of the differing abilities of each strain to respond to the immunogen haIgG]. However, C3H mice receiving F/T_{17} show no suppression.

Discussion

The suppression transferred by F/T_{17} extract is genetically restricted. Therefore, the suppressive factor(s) present in F/Tl7 extract must carry information pertaining to the MHC in addition to information on the antigen that induced the factor(s) in the first place.

These findings are consistent with reports by other workers of suppressive factors derived from T cells that bear determinants coded for by the I-region genes of the major histocompatibility complex (MHC) have been described by several workers including, Tada et al (183), Theze et al (190), Moorhead (193), Feldman et al (194) and Taniguchi et al (135); the latter for a HGG specific suppressive factor.

CHAPTER V

General Discussion

It has been demonstrated that HIgG(mPEG)$_n$ conjugates (where n = 5,13,17 or 20) can induce specific suppression at doses of 10 or 100 micrograms in female BDF$_1$ mice. A requirement for `close association' of protein and mPEG was demonstrated by the lack of suppression following injection of an admixture of HIgG and hydrolysed intermediate. The amount of suppression induced was related both to the dose of conjugate administered and to the degree of substitution, n.

These results are consistent with those of Lee and Sehon (154-157) who demonstrated the tolerogenic properties of OA(mPEG)$_n$ conjugates; and of Wie et al (163) who demonstrated the tolerogenic properties of conjugates of PEG with OA, Timothy grass pollen, Dog albumin and bovine pancreatic ribonuclease. That the tolerogenic properties of HIgG(mPEG)$_n$ conjugates were not a unique feature of the human myeloma (designated HIgG#1) used to synthesise HIgG(mPEG)$_{20}$ was demonstrated by the tolerogenic properties exhibited by HIgG(mPEG)$_{17}$, synthesised from a second human myeloma (designated HIgG#2). The suppressive properties of HIgG(mPEG)$_{17}$ were tested in vivo by the i.v. and i.p. routes and found to be most potent when administered i.v..

The source of immunoglobulin used in tolerance induction studies may be important; thus Parks and Weigle (127) studied the tolerance

induced by DHGG derived from several different sources (from commercial pooled Cohn fraction II, from the plasma of a healthy patient and from the plasma of a myeloma patient). Although the level of transferable suppression varied, all of the DHGG preparations purified from commercial sources were capable of inducing suppressor cells whereas suppressor cells could not be demonstrated in mice tolerised with DHGG derived from the plasma of a healthy patient or with DHGG derived from the plasma of a myeloma patient. Thus suppressor cells could only be demonstrated after tolerisation of mice with DHGG derived from pooled outdated human plasma. Parks and Weigle ascribed the inability of DHGG derived from individual human plasma to induce detectable suppressor cells to the fact that these preparations were fresh and not pooled (i.e., not extensively denatured). Takatsu and Ishizaka (195) have shown that denatured antigen induces suppressor cells. The myelomas used in the present study were not freshly obtained having been stored frozen for several years.

In order to model the possible clinical application of these conjugates, the suppressive effect of multiple injections of $HIgG(mPEG)_{17}$ was studied in mice that were immunised every day for 14 days. Monoclonal antibodies used in therapy for human cancers are generally given as a course of injections or infusions i.e. their bodies are receiving several immunogenic stimuli to which they may respond by the production of anti-MAb antibodies. These therapeutically administered MAbs are not deliberately aggregated as

was the immunogen in the present study; thus, the ability to suppress despite multiple immunisations with haIgG is a more stringent test of tolerogenic potency of the conjugate than multiple immunisations with HIgG per se. It was shown that 14 injections of 100 micrograms of $HIgG(mPEG)_{17}$ could suppress the immune response to 14 immunising injections of haIgG by 91%. Suppression of the IgM response was less dramatic (approximately 60%) but the titer of IgM anti-HIgG antibodies was equivalent to only 5% of HIgG specific antibodies of the IgG isotype (neglecting consideration of different specific activities of the alkaline phosphatase coupled goat anti-mouse antibodies used to detect these two isotypes in the ELISA).

Lee and Sehon (156) demonstrated that multiple administrations of $OA(mPEG)_n$ could markedly suppress the ongoing IgE anti-OA response. Thus, mice receiving 3 daily i.v. administrations of 0.8 mg of $OA(mPEG)_6$ 37,38 and 39 days after sensitisation to DNP-OA, resulted in a marked decrease in the anti-DNP and anti-OA IgE responses following secondary and tertiary immunisations.

Some bio-physical characteristics of the suppressive freeze/thaw extracts derived from splenic leukocytes of donor mice tolerised with 100 micrograms of either $HIgG(mPEG)_{20}$ or $HIgG(mPEG)_{17}$ (designated F/T_{20} and F/T_{17} respectively) were studied. The suppressive factor(s) present in the F/T extracts had a molecular weight in the range 13.7-25 kD. This implies that HIgG (molecular weight approximately 160 kD) from the $HIgG(mPEG)_{20}$ conjugate used to tolerise the donor, mice was not associated with the suppressive

factor(s) present in the F/T_{20}.

These extracts appeared to be derived from Lyt-2.2+ sIg- cells which is consistent with these cells being effector T suppressor cells.

The suppressive factor(s) present in F/T_{20} were at least in part composed of protein because the suppressive capacity of the extract could be reduced by incubating the extract with either soluble or insoluble Protease before injection into naive syngeneic recipients. Incubation of the extract with RNAse or with DNAse did not reduce suppressive capacity.

The suppression induced by the suppressive factor(s) present in F/T_{17} could be reduced by incubation of the extract with HIgG#1, HIgG# or BIgG but not by incubation with anti-sera to any of these three immunoglobulins. This implies that (a) the factor(s) recognise some common determinant present on the immunoglobulins; (b) HIgG from the $HIgG(mPEG)_{17}$ conjugate used to tolerise the donor mice was not associated with the suppressive factor(s) present in the F/T_{17}.

F/T_{17} was unable to transfer suppression across MHC differences i.e. F/T_{17} derived from BDF1 (c57BL/6 x DBA/2) mice (haplotype: b/d) could induce suppression into either parental strain i.e.,C57BL/6 (haplotype: b) or DBA/2 (haplotype: d), but not into C3H mice (haplotype: k). All strains could be tolerised by direct administration of $HIgG(mPEG)_{17}$ although to differing degrees.

The production of protein-containing, antigen specific, tolerogenic extracts from sIg-, Lyt-2.2+ suppressor T cells is

consisitent with the findings of Jones and Kaplan (134) and of Taniguchi and Miller (135). However, the molecular weight estimate for the extracts produced in the present work was in the range of 13.7 - 25 kD compared with a range of 30-55 kD for the HIgG suppressive factor obtained by Taniguchi and Miller. and approximately 45 kD for the factor described by Jones and Kaplan (134). The molecular weight range obtained in the present study is closer to that determined by Sehon and co-workers (162) for a suppressive factor with a molecular weight in the range 20-50 kD derived from cells of mice tolerised with DNP-PVA conjugates. The fractionation profiles of the HIgG specific suppressive extracts produced in the present work differed from the HGG specific suppressive factor of Jones and Kaplan (134) which was in turn different from that of Taniguchi and Miller (135). In other respects the three suppressive factors or extracts were similar (with respect to cells of origin, protein content, specificity and antigenic determinants). It is unclear whether or not the apparent molecular weight differences reflect truly different suppressive molecules. The problems inherent in the method of producing the F/T extracts was discussed in chapter 4. The culturing of HIgG$(mPEG)_n$ tolerised cells may lead to the secretion of suppressive factors (i.e., a cleaner system in comparison with freezing and thawing sIg- cells) in sufficient quantity to allow detailed bio-physical characterisation. It is possible that more than one suppressive factor is responsible for tolerance.

The specificity of the tolerance induced by HIgG(mPEG)$_{17}$ was found to extend to other immunoglobulins (HIgG#1 and BIgG) but not to OA. In addition, all three of these proteins were incapable of terminating HIgG specific tolerance. Benjamin (141) demonstrated that mice tolerised with DHGG were not only tolerant to HGG but also to BGG and postulated that the determinants recognised on HGG and BGG by HGG induced suppressor cells may be essentially identical. Ruben et al (174) studying DHGG induced tolerance in A/J mice, suggested that extensive cross-reaction between gamma globulins at the T cell level could explain the cross-tolerance observed when mice tolerant to HGG were immunised with heterologous gamma globulins. The cross-reactivity demonstrated in the present study is reflected in the property of BIgG (and another human myeloma, HIgG#1) to reduce the suppressive ability of F/T$_{17}$ extract if they are incubated with the extract before injection into recipient mice i.e., the F/T$_{17}$ recognises determinant(s) which are similar in the three immunoglobulins.

Cross-tolerance has implications for clinical applications of XIgG(mPEG)$_n$ conjugates because it may not be necessary to custom synthesise conjugates for every therapeutic MAb or MAb-toxin in clinical use.

Proteins that are more distantly related to the tolerising protein (e.g., non-mammalian immunoglobulins or immunoglobulins derived from the Monotremata or Marsupialia) are generally more effective at terminating tolerance (196,197). Thus, it is possible

that the response to aggregated non-mammalian gamma globulins or aggregated gamma globulins derived from the two sub-classes mentioned above may not be suppressed by pretreatment with $HIgG(mPEG)_{17}$ and that such immunoglobulins may also be capable of terminating HIgG specific tolerance. This would not present a problem in the clinical application of these conjugates if both the tolerogenic conjugate and the therapeutic MAb are both of murine origin or both of human origin. However, use of MAb of human origin with tolerising conjugate of murine origin (or vice-versa) may present a problem with regard to loss of cross-tolerance. This may depend upon the genetic distance (i.e., how different are the immunodominant determinants present on immunoglobulins of the two species) between human and mouse gamma globulins compared with that between human and cow which is sufficiently small to allow cross-tolerance to operate.

The occasional observation of suppression in recipients of splenic leukocytes from naive (i.e., given PBS in place of conjugate) donor mice that are subsequently immunised with haIgG, may be a consequence of the special nature of immunoglobulins when they are used as antigens. In contrast this was not observed with control mice and their response to AOA. Immunoglobulins, perhaps, should not be considered merely as passive protein antigens because they are themselves functional parts of the immune system albeit a xenogeneic one. Immunoglobulins possess idiotypic, allotypic and isotypic determinants in addition to complement binding domains. They are also able to bind to Fc receptors of certain cells. It is possible

that the human myelomas used in the present work could interact with the host's immune system not simply as passive antigens but also as active functional immunoglobulin due to cross-reactivity with host cell receptors et.c.. The well documented observation that deaggregation of immunoglobulin renders it tolerogenic, whereas aggregation renders it immunogenic (76) lends support to the supposition that immunoglobulins are unique amongst protein antigens.

Xenogeneic immunoglobulin may be envisioned as interacting not only with various anti-idiotypic, anti-allotypic and anti-isotypic clones of cells, but also through its Fc moiety and possibly via complement and C3 receptors on cells. This multi-facetted interconnection may facilitate unique juxtapositioning of cells and/or of the xenogeneic immunoglobulin on any given cell: in general immune complexes which bind to B cells via Fc and antigen receptors result in inhibition of that clone. Antigens complexed with antibody show greatly increased localisation in germinal centers. In general IgM complexes result in augmentation of the immune response whereas IgG comlexes result in suppression.

Innate regulatory networks existing in naive mice which regulate autochthonous Ig may be perturbed by the introduction of xenogeneic immunoglobulin due to the close resemblance of the latter's determinants to self-determinants. Thus, the host's immune system may be unable to distinguish certain xenogeneic determinants from certain autochthonous determinants. The xenogeneic Ig will presumable interact with idiotype specific clones and perturb the

idiotype-antiidioype network of the host resulting in suppression.

Mechanism of tolerance

Studies on the kinetics of tolerance induction with 100 micrograms of HIgG(mPEG)$_{20}$ showed that conjugate could induce tolerance if it were administered from 6 to 43 days prior to immunisation. Administration one day before immunisation gave suppression but to a lesser degree. Administration of conjugate immediately before or 6 hrs or 63 days before immunisation had no significant effect. In contrast enhancement of the immune response was observed when HIgG(mPEG)$_{20}$ was administered 2 days after immunisation.

These results are in general agreement with those for tolerance induction with DHGG of Parks and Weigle (127) and of Chiller et al (125) with respect to the minimum time required to induce optimal suppression. However, the duration of tolerance reported by Chiller et al (125) was longer (120 days for thymocytes and 50 days for B cells). The difference in duration of tolerance in the present work (43 days) compared with the that reported by Chiller et al (125) may be due to the fact that these workers used high doses of DHGG (2.5 mg) compared with 100 micrograms in the present work, as the induction of unresponsiveness to HGG is dose dependent (120).

Specific tolerance could be transferred into naive syngeneic recipients by splenic leukocytes, sIg- splenic leukocytes and freeze/thaw (F/T) extracts of sIg- splenic leukocytes derived from

donor mice tolerised with 100 micrograms of $HIgG(mPEG)_{20}$ or of $HIgG(mPEG)_{17}$.

The observation of occasional (as opposed to consistant) suppression in naive mice used in transfer experiments may be a result of the genetic inhomogeneity of the strain of mice used in the present work. These mice are female BDF_1 and are the first generation progency of C57BL/6 x DBA/2 inbred mice strains. Thus any individual mouse is not necessarily homozygous at every genetic locus with another BDF_1 individual. In this instance, a recipient BDF_1 mouse may well recognise biologic materials derived from one of its BDF_1 relatives as foreign, irrespective of whether the donor mouse had been treated with conjugate previously.

Further fractionation of sIg- leukocytes derived from donor mice tolerised with 100 micrograms of $HIgG(mPEG)_{17}$ into antigen positive (sIg-/rHIgG+) and antigen negative leukocytes (sIg-/rHIgG-); and comparison with other tolerant cell populations of the number of cells required to transfer tolerance yielded the following ranking of tolerogenic potency: sIg-/rHIgG+ > sIg- > sIg-/rHIgG- > unfractionated leukocytes. Tolerance was not transferred by the sIg+ cell population of leukocytes derived from donor mice tolerised with $HIgG(mPEG)_{17}$ or $HIgG(mPEG)_{20}$.

In the present work the procedures employed for enrichment of cell populations, i.e., sheep anti-mouse Ig and HIgG coated petri dishes, resulted in approximately 10 fold enrichment. Thus, all T suppressor cells were not depleted by incubation on plates coated

with each of the above named immunoglobulins.

Radiolabelled derivatives of HIgG$(mPEG)_{20}$ demonstrated that this transfer of tolerance was not due to simple carry-over of tolerogen as the amount of tolerogen transferred was 100-1000 fold less than that required to induce tolerance.

The possibility that the amount of radioactivity detected in recipients does not necessarily reflect the amount of tolerogen must be considered. It is possible that the radiolablel was excised from the rest of the tolerogen (or derivative thereof) in vivo and that there is no correlation between cpm and amount of conjugate present.

The results of experiments whereby the putative suppressor factor (freeze/thaw extract) were incubated with anti-HIgG antibodies and yet were still able to induce suppression in recipients, argues against conjugate carry-over (if conjugate carry-over were responsible for suppression induction in the recipient mice, then incubation with anti-HIgG antibodies should deplete the freeze/thaw extract of conjugate and reduce or abrogate suppression). However, it is possible that the attachment of mPEG groups to HIgG may reduce the latter's antigenicity, and therefore also reduce conjugate/anti-HIgG antibody interactions.

The cell population responsible for transferable tolerance was shown to be Lyt-2.2 positive. This is consistent with this population being T suppressor cells.

The duration of transferable tolerance was compared with the duration of tolerance in intact mice; and found to be approximately

15 days following administration of 100 micrograms of $HIgG(mPEG)_{20}$ compared with approximately 43 days in intact mice. The difference in kinetics between transferable tolerance and tolerance in the intact mouse suggested that T suppressor cells whilst capable of transferring suppression into naive syngeneic recipients may not be relevant to the maintenance of tolerance in the intact mouse. However, it was possible that the suppressor T cells were present in two distinct populations; initially as T suppressor cells for a period of approximately 15 days and then as T suppressor memory cells, which were only functionably detectable when the animal was re-exposed to immunogen.

One hundred micrograms of conjugate administered i.v. into BDF1 (C57BL/6 x DBA/2), was used to induce transferable tolerance in the present study. Weigle et al (198) have shown that 100 micrograms of deaggregated HGG (DHGG) induces specific tolerance in T cells but not in B cells. However, the amount of DHGG required to induce tolerance varies for different strains of mice; thus 100 micrograms of DHGG can induce tolerance in C57BL/6 mice but not in BALB/c mice (120). (The ease or difficulty of induction of tolerance to DHGG is under genetic control. Thus, when cross-matings between C57BL/6 and other strains were made, the dose required to render the F1 mice unresponsive was similar to that required for the C57BL/6 parent (120)). The BALB/c mice may process the small amount of aggregated material known to remain in ostensibly de-aggregated (ultracentrifuged) HGG, sufficiently quickly that they become sensitised before tolerance can

be established. Conversely C57BL/6 mice may process the aggregated material in an inefficient, slow manner such that tolerance is established before the mice can be sensitised. Stumpf et al (199) using low doses of BSA have demonstrated that low zone tolerance is maintained by T suppressor cells and have postulated that any foreign (not self cross-reacting) determinant entering the immune system in a limiting dose will predominantly activate T suppressor cells resulting in their functional dominance. This preferential activation of suppressor T cells over helper T cells may be related to a lower activation threshold of the former cells (200). Weigle (201) using cell transfer methods, demonstrated that low zone tolerance corresponds to a net unresponsiveness in the helper T cell population, whereas high zone tolerance reflects unresponsiveness in both the helper T cell population and in the B cell population.

Benjamin (141) induced tolerance in A/J mice by the injection of 100 micrograms of DHGG given i.p. but could not demonstrate suppressor cells in his adoptive transfer system. However this does not rule out the induction of suppressor cells by low doses of DHGG but may reflect the insensitivity of the suppressor cell detection system, because, as discussed previously in chapter 3, the use of irradiated recipients may work against the transfer of suppression (175).

Evidence for the presence of suppressor cells in the present work in mice tolerised with 100 micrograms of $HIgG(mPEG)_{20}$ and of $HIgG(mPEG)_{17}$ includes the following findings: (a) the demonstration

of transferable suppression by tolerised mice into naive syngeneic recipients, (b) the demonstration that transfer of tolerance is not due to carry-over of tolerogen, (c) the demonstration that transfer of tolerance can be mediated by the sIg- (T cell enriched) cell population and (d) the demonstration that treatment of tolerant spleen cells with anti-Lyt-2.2 MAb plus complement, can reduce their suppressive ability. The induction of suppressor T cells is consistent with the findings of Sehon and co-workers (156,160,161) using $OA(mPEG)_n$, and for tolerance induced by deaggregated heterologous gamma globulins by Parks and Weigle (127), Basten et al (129), Benjamin (130), , Doyle et al (137), Jones and Kaplan (134) and Weigle et al (202). The suppressor cells induced by DHGG was shown to be a T lymphocyte by Basten et al (129) and Doyle et al (131) and to be Ly-2+ by Vadas et al (132) and by Taniguchi and Miller (133); as were the suppressor cells induced by $OA(mPEG)_n$ (161)

However, in the present work the kinetics of transferable suppression did not correlate with those for suppression in the intact animal. Similar observations have been made for DHGG induced suppressor cells by Basten et al (131), Benjamin (130) and Doyle et al (131). As a consequence of these latter observations Doyle et al (131) and Parks and Weigle (127), consider the production of suppressor T cells to be coincidental with, but not obligatory for, the induction of tolerance by DHGG. This view has been countered by Loblay et al (142) who has demonstrated the existence of two distinct sets of suppressor cell; the familiar T suppressor cell and a memory

T suppressor cell which is detectable only following secondary antigenic stimulation. Loblay et al (142) were able to demonstrate memory suppressor cells in mice given as little as 10 or 100 micrograms of DHGG given i.p. into in CBA/Ca/T6 mice. (Loblay et al also demonstrated suppressor memory cells in mice receiving high doses of tolerogen i.e., two 3 mg doses of DHGG given 60 days apart).

In the present work no suppressor memory cells were detected in mice given two doses of 100 micrograms each of $HIgG(mPEG)_{20}$ 57 days apart and their splenic leukocytes transferred into naive syngeneic recipients 2, 4 or 6 days later. Recipients of splenic leukocytes derived from donor mice that had been injected with conjugate 6 days prior to sacrifice do show suppression when challenged with haIgG. This refutes the suggestion that the tolerogenic material is sequestered in an anatomic site other than the spleen in the donor mice; and that the transfer of inappropriate cells is the reason for the lack of demonstrable suppression memory in the transfer experiments discussed elsewhere.

The presence of memory suppressor cells was investigated by attempting to demonstrate suppression memory in mice given two widely separated injections of conjugate, by looking for the classical attributes of immunological memory i.e., more rapid induction of tolerance, more profound tolerance and increased duration of tolerance. However, using two injections of 100 micrograms each of $HIgG(mPEG)_{20}$ given 57 days apart (i.e., an interval previously demonstrated to be sufficiently long to allow the tolerance induced

by the first injection of conjugate to be waning) no evidence for suppressor memory cells was obtained. Benjamin (141) was unable to demonstrate the reappearance of suppressor cells in mice given a second dose to DHGG 80 days after the first dose which had produced suppressor cells.

Recipient mice were immunised the day after injection of the cells. This differs from Loblay's method in that his mice were tolerised with DHGG and immunised 42 days later. Five days after immunisation these mice were sacrificed and their spleen cells transferred into irradiated recipients. Thus, the mice in Loblay's experiments received an immunogenic stimulus 5 days before transfer as opposed to one day after transfer in the present work, allowing sufficient time for the activation of memory T suppressor cells before transfer and therefore the availability of sufficient numbers of T suppressor cells to shift the immunological equilibrium in favour of suppression. Prolongation of the tolerant state by subjecting tolerised animals to an immunogenic stimulus has been reported by Fidler and Golub (203), Elson and Taylor (204) and Bell et al (205). It is, therefore, possible that immunising recipient mice one day after transfer rather than immunising donor mice five days prior to transfer may account for the absence of evidence for suppression memory in the present work. The transfer of splenic leukocytes into naive syngeneic recipients places the putative T suppressor cells (and factors) into an immunological environment where the host's unprimed HIgG reactive Ts, Th or B cells may

dominate. This contrasts with the admininstration of two separate injections of conjugate to the same mouse where such cells are already primed.

The role of mPEG in the conversion of HIgG to a tolerogen

The increase in suppressive ability with increase in the degree of substitution may be related to the reduction in the capacity of HIgG to aggregate as a result of increasing the number of mPEG molecules per HIgG molecule and/or by shielding of antigenic determinants by the mPEG chains.

(a) Decrease in aggregated material

Suzuki et al (167) demonstrated that as the number of PEG chains coupled to IgG increased, the aggregatability of the conjugate decreased. It has been postulated that PEG forms a flexible hydrophilic shell around the protein to which it is conjugated (150). Thus, enzyme$(PEG)_n$ conjugates are soluble over a wide range of pH compared with the unconjugated enzyme and enzyme$(PEG)_n$ conjugates partition into the PEG phase of dextran and PEG two phase systems. Abuchowski et al (149) have reported that enzyme$(PEG)_n$ conjugates above a certain degree of substitution are not held on either cationic or anionic exchange columns. In addition ultracentrifuged enzyme$(PEG)_n$ conjugates sediment at a slower rate than the unconjugated enzyme (150) possibly due to hydrogen bonding by water molecules to the ether oxygen atoms of the PEG

The attachment of PEG to proteins may effect the ability of macrophages to phagocytose and/or process them. Golub and Weigle (120) reported that the slow phagocytosis of DHGG (compared with the faster processing of aggregated HGG) contributes to the induction of tolerance to HGG. Work in this laboratory on comparing the uptake of radiolabelled $OA(mPEG)_n$ with that of radiolabelled OA suggested (unpublished observations) that the conjugate was taken up by adherent cells to a lesser extent than OA in vitro. In the present work comparison of the uptake of radiolabelled $HIgG(mPEG)_{20}$ with that of radiolabelled sham conjugated HIgG was equivocal (data not shown).

In vivo studies showed that $HIgG(mPEG)_{20}$ remained in circulation at levels six fold greater than those of sham conjugated HIgG and that there was no accumulation of conjugate in organs of the mouse. These results are consistent with those of Davis et al (150) who showed that PEG conjugates of various enzymes (e.g., arginase, catalase and uricase) remain longer in the animal than the unconjugated enzyme and that these conjugates did not accumulate in organs of the test animals.

The introduction of 20 mPEG side chains to the HIgG molecule does not alter its rate of catabolism. This suggests that the recognition of xenogeneic immunoglobulin by phagocytes and by the active sites of catabolic enzymes is not affected by the presence of the mPEG chains attached to the HIgG backbone.

Dresser (72,76,78-98,97) demonstrated that the physical nature of the antigen determined the immunological outcome of administration of

heterologous gamma globulin to mice. Deaggregated gamma globulin induced tolerance at low doses (10-200 micrograms) whereas aggregated material induced an immune response. Chiller and Weigle (206), Biro and Garcia (207) and Claman (208) also reported tolerance induction by ultracentrifuged proteins. Golub and Weigle (120) and Frei et al (209) were able to achieve a similar separation of antigen into immunogenic and tolerogenic components by salt fractionation and gel filtration respectively. Golub and Weigle (120) also demonstrated in vivo fractionation (`biological filtration') of HGG by passage of the material through animals; the aggregated material being rapidly phagocytosed and the non-aggregated (tolerogenic) material could be recovered by bleeding the animal. Similar `biological filtration' has been reported by Geyere and Kong (210) and by Frei et al (209). Thus certain proteins can be fractionated by physical means into immunogenic (aggregated) and tolerogenic (non-aggregated) components.

However, aggregated antigen preparations can in some instances induce tolerance rather than an immune response. For example, Dvorak et al (211) using human serum albumin in Guinea pigs and Birnbaum et al (212) using DNP-BGG in a murine model.

(b) Shielding of helper determinants

Antigen systems have been described in which the antigen possesses both helper and suppressor determinants. This has been demonstrated in a murine model for Hen Egg Lysosome (HEL) and for related lysozymes by Sercarz et al (213). A suppressor determinant on HEL

which contains phenyalanine in the third amino-terminal position is recognised by C57BL/6 mice, leading to suppression. Replacement of phenylalanine by tyrosine converts the suppressor determinant into a helper determinant. In addition, a functionally silent helper determinant coexists with this suppressor determinant on the same molecule located between amino acid positions 13 and 105. Other examples of the coexistence of helper and suppressor determinants in cellular antigens have been reported by Klein et al (214) in a tumour cell line, by Chan and Henry (215) in carrier primed spleen cells and by Frost et al (216) in a murine tumour abrogating the generation of cytotoxic T cells. In these systems the suppressor determinant on the antigens is dominant, the coexisting helper determinant is silent but can be detected if the suppressor determinant is removed.

It is possible that the covalent attachment of the relatively long chains of mPEG (average molecular weight 6,548) to HIgG may lead to the masking of helper determinants and/or to the exposure of suppressor determinants as a result of conformational shifts induced by hydrophilic interactions between mPEG and hydrophilic regions of the protein molecule and/or the carbohydrate moiety of the HIgG. However, it is not clear why helper determinants should preferentially be hidden by mPEG, whilst these same mPEG groups have no effect upon the rate of HIgG$(mPEG)_{20}$ catabolsim in vivo. [see above].

However Suzuki et al (167) using circular dichroism and immunoelectophoretic analysis detected no alterations in the

secondary or tertiary structure of the IgG molecule after the covalent attachment of 25 PEG groups.

Conclusion

The data presented here show that XIgG(mPEG)$_n$ conjugates are tolerogenic at certain doses and with certain n values. The latter observation implies that the amount of mPEG attached to the XIgG molecule determines its tolerogenicity at a given dose. The means by which mPEG exerts this effect is not clear. Evidence for a role in limiting the degree of aggregation and for masking of helper determinants was discussed above. Evidence has been presented for a role of Ig specific Lyt 2.2+ suppressor T cells in the maintenance of tolerance induced by XIgG(mPEG)$_n$ conjugates. Application of Occam's razor to a consideration of the available data from the present study and from analogous systems, leads to the conclusion that the attachment of mPEG to XIgG yields conjugates that are protected from aggregation. This low amount of aggregation results in slow clearance in vivo by macrophages thereby favouring the induction of tolerance. Other mechanisms may also be operative.

The clinical viability of XIgG(mPEG)$_n$ conjugates remains to be determined. The requirement for tolerance induction in some patients undergoing MAb or MAb-toxin treatment was demonstrated in chapter 1. The deaggregation of these MAbs immediately prior to administration is not a practical means of tolerance induction because the adoption of such a strategy would require access to ultracentrifugation

equipment, technicians to operate the equipment, exact timing of treatments and relatively large quantities of the MAb since only the upper two-thirds of a tube of ultracentrifuged material could be injected (i.e., the aggregated material separates into the lower third).

In the present work suppression was of the order of 95% but did not reach 100%, with respect to the IgG and IgG1 response to haIgG. In actual clinical practice the therapeutic MAbs administered to patients will not be deliberately heat-aggregated as in the present work. thus any tolerogenic conjugate given before MAb therapy begins will not be challenged to the same degree. It is not known whether the residual 5% (if it is also observed in the human) would prevent MAbs from achieiving their objectives in vivo.

The lack of suppression of the IgM anti-haIgG response in mice treated with IgG conjugates may imply that the conjugate share the isotype of the therapeutic MAb.

The use of suppressive factors derived from deaggregated immunoglobulins could circumvent these requirements. Large quantities of recombinant factors could be commercially manufactured in the future. However, the possible long term instability of such factors may limit their usefulness.

In addition the factors themselves could be immunogenic especially in the case of recombinant derived materials which often differ subtely from their natural in vivo counterparts

By contrast, XIgG$(mPEG)_n$ conjugates do not require deaggregation in

order to induce tolerance and therefore could be available for use at any time and do not require specialised (and costly) manpower and equipment.

Original contributions

1. Synthesis of HIgG(mPEG)$_n$ conjugates (where n=5, 13, 17 or 20) capable of inducing specific suppression at doses of 10 or 100 micrograms.

2. The amount of suppression induced was related to both the dose and degree of conjugation (n) of conjugate administered.

3. Administration of 100 micrograms of the n=20 conjugate 6 to 43 days prior to immunisation induced specific tolerance. Longer periods (63 days) or shorter periods (6 hours) prior to immunisation resulted in no suppression.

4. Multiple i.v. injections (5, 7 or 14) of 100 micrograms of the n=17 conjugate resulted in suppression of the immune response to 14 daily immunisations. The degree of suppression is related to the number of injections of conjugate.

5. Mice treated with 100 micrograms of HIgG(mPEG)$_{17}$ were able to suppress the anti-haIgG#1 and the anti-haIgG#2 responses and to also the anti-haBIgG response, although to a lesser degree.

Mice treated with 100 micrograms of conjugate and immunised with either haIgG#1, haBIgG or AOA all showed suppression with respect to the anti-HIgG response i.e., the tolerance induced by the conjugate could not be broken by immunisation with these proteins.

6. Specific suppression is transferable into naive syngeneic recipients by Lyt-2.2+, sIg- splenic leukocytes derived from donor mice treated with 100 micrograms of $HIgG(mPEG)_{20}$ or with 100 micrograms of $HIgG(mPEG)_{17}$.

7. Suppression in intact mice induced by 100 micrograms of $HIgG(mPEG)_{20}$ lasted for 43 days compared with only 15 days for transferable suppression i.e., suppression could be transferred by splenic leukocytes derived from donor mice treated with 100 micrograms of $HIgG(mPEG)_{20}$ into naive syngeneic recipients for a period of up to 15 days following administration of conjugate to the donor mice.

8. Specific tolerance is transferable into naive syngeneic recipients by freeze/thaw extracts of Lyt2.2+, sIg- splenic leukocytes derived from mice treated with 100 micrograms of conjugate.

9. The freeze/thaw extracts contained a suppressive moiety that was at least in part proteinaceous, specific for HIgG#1, HIgG#2 and BIgG and was H-2 restricted with respect to transfer of tolerance.

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