The University of Manitoba

# REGULATION OF IgE ANTIBODY PRODUCTION IN NORMAL AND IRRADIATED B6D2F1 MICE

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A thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

Department of Immunology

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ABSTRACT OF THESIS

An adoptive cell transfer system was defined for studying the long-term IgE anti-ovalbumin (OA) response of B6D2F1 mice. The primary IgE anti-OA response given by this strain persisted for more than 12 months yet the half-life of IgE antibody in circulation was only 10.5 hours as measured by residual passive cutaneous anaphylaxis (PCA) in rat skin. The system developed to study the cell recruitment patterns which were thought to underlie this persistent response involved the transfer of primed spleen cells to irradiated recipients and the production in these recipients of IgE and hemagglutinating (HA) antibody without further overt antigenic challenge. The capacity of primed spleen cells to transfer the response was first detectable 10 days after immunization, increased for the next 6 weeks and was still evident 8 months after immunization of the donor animals. The transferred response itself persisted in the recipients for many months.

The anti-OA response in recipient mice was inconsistently dependent on the presence of fetal calf serum (FCS) in the medium in which the cells were suspended. Primed spleen cells suspended in serum-free medium containing lipopolysaccharide (LPS) also transferred the anti-OA response without requiring antigenic challenge of the recipients. No antigenic similarity could be shown to exist between OA, FCS and LPS in either PCA or HA assay. Anti-OA antibody injected into irradiated recipients immediately before transfer of primed cells suppressed the production of both IgE and HA antibodies to OA in the recipients. This implied that antigen

was present in cell-bound form on primed cells.

Depletion of macrophages from the donor spleen cell suspensions did not diminish the capacity of the remaining cells to give an adoptive response. The production of IgE antibody in irradiated recipient mice was prevented if donor B or T lymphocytes were treated in vitro with either gamma irradiation or mitomycin C, suggesting that proliferation of both B and T lymphocytes was essential for the adoptive response to develop.

However, the requirement for proliferation was only transient since, once IgE antibody production reached a steady state in the recipients, it manifested extreme resistance to high dose irradiation. Whole-body irradiation of 800 and 1,000 rads was without effect on established IgE production. This latter observation was valid for both intact mice which were irradiated 8 weeks after immunization and also for irradiated adoptively immunized mice. These data suggested that the IgE anti-OA antibody measured in serum of BDF1 mice several months after immunization with 1 μg OA and 1 mg $Al(OH)_3$ was primarily the product of long-lived antibody-secreting cells.

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# CHAPTER 1: INTRODUCTION

## 1.1 Evidence for the existence of Immunoglobulin E

Evidence for the existence, in plasma, of a factor capable of mediating an immediate hypersensitivity response was first reported by Prausnitz and Kustner in 1921 (1). A small volume of serum taken from the allergic Kustner was injected into the skin of Prausnitz. When the injection site was subsequently challenged with a small amount of fish extract, to which Kustner was sensitive, a reddening and swelling of the area resulted. This method of passive sensitization to test for the presence of reagin has since been called the P-K test in recognition of the authors and was central to the eventual isolation and characterization of IgE.

Investigators during the next few decades identified some of the physico-chemical properties of the reagin. The skin sensitizing activity was found associated with the globulin fraction of serum (2,3), had a sedimentation coefficient of 7.8S and, on electrophoretic analysis, migrated with the proteins in the beta - gamma region (4). By 1963 antigenic analysis of the structure of antibodies had identified three classes of immunoglobulin designated IgG, IgM and IgA and, by 1965, a fourth class, IgD, had been identified (5). For a time data were reported which associated reaginic activity with IgA (6,7). However, the identification of reagin in an individual deficient in IgA (8) and the inability of anti-IgA serum to neutralize reaginic activity (9,10) pushed Ishizaka and his colleagues to pursue the isolation of

a separate class of immunoglobulins.

Ishizaka et al isolated reagin by extracting it from the serum of people with hay fever. The presence of reagin in the fractions was determined by monitoring skin-sensitizing activity. These investigators then immunized rabbits with the reagin-enriched fraction. The resulting antisera were used to compare the antigenic structure of reagin to the other known immunoglobulin classes (9,10,11). Analysis by double-diffusion in agar gel of reagin revealed the presence of both kappa and lambda light chains, as in the other known immunoglobulin isotypes, and of antigenic determinants not shared by IgG, IgM or IgA. The apparently new class of immunoglobulin was designated IgE (12). Mixing of this IgE globulin with reaginic serum prevented skin sensitization by the latter. This observation was interpreted to mean that the two competed for a single biological target which implied similarity if not identity of the two materials. In addition, Ishizaka et al were able to demonstrate that the titre of reaginic activity, as determined by skin testing, correlated with the amount of IgE in the test preparation but not with the quantities of IgG, IgM or IgA present (13). This further strengthened the postulate that immediate hypersensitivity responses were mediated by a distinct immunoglobulin class.

At about the same time, Johanssen and Bennich identified an atypical myeloma immunoglobulin from a patient identified as ND (4). On reaction with the anti-reagin serum

which had been prepared by Ishizaka, IgND was shown to be antigenically similar to IgE. Within a year of this collaborative effort the World Health Organization had officially designated this IgE as a new class of immunoglobulin (14).

## 1.2 Physico-chemical properties of IgE

To date no subclasses of IgE have been identified. The existence of human IgE-secreting myelomata permitted isolation of sufficient quantities of this immunoglobulin to allow more precise characterization of the molecule (15,16). IgE is a glycoprotein with a relative molecular mass of 190,000 and a sedimentation coefficient of 8S. In common with other classes of immunoglobulin, it is composed of two identical heavy chains (H), designated epsilon, and two identical light chains (L) which were either kappa or lambda. Each epsilon chain has five recognizable domains and each light chain two. Domains are compact, globular arrangements of approximately 110 amino acids each organized into a 60 residue loop by an intra-chain disulphide bond. Their existence was first suggested by the linear periodicity of the amino acid sequences of immunoglobulins (17). The four chains are held together by disulphide bonds. One such bond exists between each of the two heavy and light chain pairs and two bind the heavy chains together. The result is a Y-shaped molecule whose arms are composed of one light chain and the amino-terminal half of the heavy chain. The stem is the

paired carboxy terminal ends of the heavy chains. Enzymatic digestion of the molecule followed by testing of the resulting fragments has shown that the two arms are the antigen binding (Fab) fragments of the antibody while the stem can be isolated as a crystallizable fraction (Fc). This basic type of structural organization was first demonstrated for IgG by Porter in 1959 and has proven to be common to all immunoglobulin classes. It is the Fc portion of the immunoglobulin which is most intimately associated with the skin sensitizing activity of IgE. It is by means of the Fc stem that the molecule binds to the membrane of mast cells and basophils (16,18) thereby "sensitizing" these cells. The two Fab sites remain exposed to the extra-cellular fluid and are free to bind antigen should it become available.

The carbohydrate content of IgE, constituting about 12% by mass, is associated with the CH1, CH2 and CH3 domains. The major sugars are mannose, N-acetyl glucosamine, galactose, fucose and sialic acid and have been shown to be essential for the binding of the molecule to cell membranes (19). The limited sequence homology (27 - 35%) between the epsilon chain and heavy chains of other classes indicates that IgE appeared early during the evolution of the immunoglobulins. Unlike other immunoglobulin classes the biological activity of IgE is impaired by moderate heat. Exposure to 56$^{o}$C for 30 minutes will destroy the skin sensitizing activity of IgE while the antigen binding activity of the molecule is maintained. This loss of biological activity has been shown to be due to

conformational changes in the Fc portion of the molecule on exposure to heat. Like other immunoglobulin classes, with the exception of IgG, IgE is reported not to cross the placenta. Plasma IgE is not in aggregated form, and hence does not activate the complement system either by the classical or the alternate pathways. However, artificially aggregated IgE does activate the alternate complement pathway (20). Ishizaka et al demonstrated that this ability to fix the late (C3 - C9) components of complement resides in the amino terminal end of the Fc portion of the molecule.

The development of radioimmunoassay (RIA) technology in the late 1960's permitted the quantitation of previously unmeasurable IgE concentrations in the circulation of normal persons (21). Polmar et al demonstrated that in a population of 73 normal adults concentrations of serum IgE were normally distributed (22). Concentrations of IgE are the lowest of all immunoglobulins and, in humans, average approximately 0.1 mg/L in the adult. Demonstration of the existence of IgE-like molecules in species other than man initially depended on the identification of skin-sensitizing activity in the sera of animals which had been experimentally immunized. Reaginic activity was found in the rat (23), dog (24), guinea pig (25), rabbit (26), mouse (27), monkey (28), cow (29) and sheep (30). Radio-immunodiffusion studies have identified the presence of IgE-like immunoglobulin in these species. Moreover, myelomata of the IgE type have been identified in a number of species. One of the more striking examples of

which is the Lou/Wsl rat strain which was identified by Bazin to be particularly susceptible to the development of spontaneous ileo-coecal immunocytomata (31). More than one fourth of such tumors have been found to secrete IgE. Studies of the properties of non-human reagins have shown them to be chemically and structurally similar to human IgE (32).

## 1.3 Biological action of IgE

In addition to existing in plasma at the lowest concentration of all the immunoglobulins, IgE has a shorter half-life in the circulation than do the other classes of antibody. In the human its half-life is 2 days (16) while IgG, IgA, IgM and IgD have half-lives of 21, 6, 5 and 3 days respectively (33). Tada has shown that in the rat the half-life of circulating IgE is 12 hours (34). However, since IgE exerts its biological activity by binding to mast cells and basophils there are two major pools of IgE in the body; circulating IgE with a relatively short half-life and cell bound IgE with a much longer half-life of approximately two weeks (35).

Tomioka and Ishizaka demonstrated the major sites of IgE binding by injecting myeloma IgE into monkeys and monitoring the localization of the immunoglobulin (36). They found that reaginic antibody bound to cells in the skin, the bronchi, the small intestine and the mesenteric membranes. These are the sites associated with the common allergic symptoms of itching, wheezing and diarrhea. The binding of

IgE to cell membranes had earlier been demonstrated in vitro. In the mid-1960's Levy and Osler showed that white blood cells taken from normal individuals could bind the reagin present in the serum of allergic individuals (37). In such early experiments the binding of reagin was, as in the P-K test, inferred from the biological consequences of exposing the sensitized cells to specific allergen. Under in vitro conditions the end-point is not the extent of tissue damage, as in the P-K test, but is a measurement of the biochemical mediators released by the senstized mast cell on exposure to antigen. The mast cell has been shown to release a number of substances the most potent of which is histamine. The major effect of histamine in the intact animal is to increase local vascular permeability causing the extravasation of fluid. On a small scale, such as in the P-K test, such a local reaction is of little consequence. However, when large areas of sensitized tissue, such as the respiratory tract, are stimulated with antigen the reaction may become life-threatening.

At about the same time Goodfriend et al demonstrated that reagin would indeed sensitize lung (38). These investigators incubated monkey lung tissue with serum obtained from hay fever patients. Subsequent exposure of the tissues to a ragweed extract triggered the release of histamine and slow reacting substance of anaphylaxis (SRS-A). Adsorption of the reaginic serum with anti-IgE reduced its ability to sensitize the lung tissue. Moreover, the release of the

mediators of anaphylaxis could be induced by exposure of sensitized tissue to anti-IgE but not by anti-IgG (39). Similar results were obtained by Orange et al using human lung as the experimental tissue (40).

The observation that anti-IgE could trigger histamine release by sensitized tissue not only confirmed that mediation of immediate hypersensitivity was a specific property of IgE but allowed further examination of the interaction of the components of the process at the cell membrane. Although intact IgE could facilitate an immediate hypersensitivity response, monovalent anti-IgE, i.e. Fab antibody fragments, could not cause the release of mediators (41). Similarly bivalent or multivalent antigens, but not haptens, were able to elicit an allergic response (42,43). These data have been incorporated into the "bridging theory" which holds that it is the cross-linking of at least two molecules of cell-bound IgE by antigen that initiates a series of cellular reactions culminating in the release of vasoactive substances. Microscopic examination has shown that the release of histamine by human basophils coincides with the degranulation of the cells (44).

In addition to the tissues already named, IgE has been shown to bind specifically to peritoneal mast cells (45) and to cultured rat basophilic leukemia cells (46). It is these last two sources that have been most extensively used to elucidate the properties of the IgE receptor itelf (47,48).

The number of IgE receptors per basophil has been

estimated to be 30,000 to 90,000. However, only 30 to 50% of these appear to be occupied by IgE molecules under normal circumstances. No difference could be found between the number of receptors in the cells of normal and atopic individuals (49). However, Ishizaka et al found that the proportion of occupied IgE receptors was greater on cells from allergic individuals than on those from normals. Moreover there was no correlation found between the amount of endogenous IgE bound to basophils and the concentration of circulating IgE. The binding of IgE to its specific receptors was found to be reversible (50). The affinity constant for the binding of IgE and its receptor was in the order of $1 \times 10^8$ to $1 \times 10^9$ L/mol for human basophils, for rat peritoneal mast cells and for cultured leukemic cells.

Despite extensive characterization of the IgE molecule and of its mode of action, the physiological role of the molecule remains unclear. There is no evidence that a deficiency of IgE leads to a functional deficit. However, there are two situations in which elevated levels of circulating IgE are associated with pathology. The first is in allergic individuals and the second is individuals infested with certain parasites. The localization of IgE on mast cells and basophils in the skin and mucosal surfaces and the release of vasoactive amines when cell-bound IgE is bridged by antigens have led to the notion that IgE functions as a gatekeeper protecting the organism from certain constituents in the external environment. The increased capillary

permeability induced by histamine would permit the extravasation of circulating antibodies and phagocytes which could then act locally to inactivate or remove a potential invader.

It is by manipulating animal models conceived to simulate the effects of allergy or parasitic infestation on IgE production that the variables controlling the synthesis of the molecule have been identified. These fall roughly into two categories. First are the isotype-specific factors which modulate circulating levels of IgE without affecting the production of immunoglobulin of other classes. The second group of factors act in an antigen-specific manner and can generally be examined in the context of the regulation of IgE antibody production.

## 1.4 Isotype specific regulation of IgE production

Helminth parasites such as Trichinella, Ascaris and Nippostrongylus clearly express adjuvant activity for the IgE response in addition to eliciting strong IgE responses against specific worm antigens. Several reports have been published to indicate that Nippostrongylus brasiliensis (Nb) in particular is a strong adjuvant for continuing IgE responses to unrelated antigens (51-53). The potentiating effects of Nb were demonstrated clearly in a study of the cellular basis for IgE production using a plaque forming cell assay for total IgE-producing cells. Following infection it was demonstrated that the level of total IgE in serum rose more than 100-fold

by approximately 16 days post infection, that most of the IgE PFC were present in the mesenteric lymph nodes, and that approximately 0.15% of the total nucleated cells in these nodes were actually producing IgE ($1500/10^6$ cells) (54). In this context it should be noted that both immunofluorescence techniques and PFC assays yielded similar numbers for IgE producing and bearing cells respectively in the mesenteric lymph nodes. With respect to spleen however, PFC assays consistently did not detect any IgE secreting cells whereas fluorescence revealed the presence of IgE bearing cells at levels somewhat greater than one tenth of those found in mesenteric lymph nodes. (55).

### 1.4.1 IgE B cell generating factor

Further study of the IgE bearing cells present in the mesenteric lymph nodes (MLN) of Nb infected rats led the Ishizakas and their coworkers to uncover the existence of cell-free factors which influenced both the numbers of IgE bearing cells and also the magnitude of the IgE antibody response. One factor was shown to cause an increase in the number of IgE bearing cells in all lymphoid tissues except the thymus. However, the induction of IgE-bearing cells after infection was not affected by neonatal thymectomy although the capacity of thse animals to produce anti-hapten antibodies against dinitrophenyl derivatives of ovalbumin was abolished (56). These results suggest that T cells are not essential for the development of IgE bearing cells into IgE antibody

secreting cells. Interestingly it was shown that the IgE bearing cells found after Nb infection synthesized their own membrane IgE. Surface IgE and other membrane immunoglobulins could be stripped off by treatment with pronase. However, after a further 24 hours in culture most of the cells regained their membrane IgE suggesting endogenous IgE production by the cultured cells (57). In the course of this extensive series of studies on the ontogeny and development of IgE bearing lymphocytes the Ishizakas and coworkers noted that IgE bearing cells increased in number not only in the peripheral lymphoid tissues but also in the bone marrow of both thymectomized and non-thymectomized animals. Since bone marrow cells do not contain a significant number of circulating lymphocytes, the possibility was considered that a soluble factor was involved in the generation of IgE bearing lymphocytes. This idea was put to the test by incubating normal bone marrow cells with cell-free supernatants derived from 24 hour cultures of mesenteric lymph node cells removed from Nb-infected rats. Thus, the existence of a factor, released from the cells of worm infected rats, and acting on normal bone marrow cells was being tested. Culture supernatants from worm infected rat lymph node cells caused a selective increase in the number of IgE bearing bone marrow cells; control culture supernatants using normal rat lymph node cells had no such effect. The factor was shown not to be simply IgE, which might have been taken up passively by the bone marrow cells. Culture supernatants passed through columns of Sepharose 4B-anti-IgE,

a treatment which would be expected to remove any IgE from the supernatants, were found still to retain their activity. Also, fractionation of the supernatants revealed that the active principle had a relative molecular mass of between 10,000 and 20,000 (58). The Ishizakas and coworkers assigned the term "IgE B cell generating factor" to the active principle and went on to show that the major source of this active material was IgE bearing B cells from the mesenteric lymph node cultures of Nb infected rats (59). A more detailed analysis of the effect of this factor on cultures of normal bone marrow cells showed that the IgE B cell generating factor converted IgM bearing virgin B cells to IgM(+)IgE(+) double bearing cells. This conversion was not inhibited by mitomycin C indicating that cell replication was not involved.

Moreover, few of the cells which became IgM(+)IgE(+) in the bone marrow cultures contained 3-H Thymidine, an observation which also was consistent with lack of cell proliferation. Treatment of the bone marrow cultures with a variety of metabolic inhibitors led to the conclusion that transcription of DNA and protein synthesis were implicated in the IgE B cell generating factor activity i.e. the conversion of IgM(+) to IgM(+)IgE(+) cells (60).

### 1.4.2 IgE potentiating factor

The thrust of subsequent work on the effects of other soluble factors produced in cultures of mesenteric lymph node cells from Nb infected rats focussed on the effect on IgE

antibody formation. For example, it was shown that when MLN from DNP-OA primed rats were cultured in the presence of T or B cells from Nb infected rats T cells from the infected rats, but not T cells from normal rats, enhanced selectively the IgE response of DNP-OA primed cells to the homologous antigen without affecting the IgG forming cell response. The enhancement of the IgE response was seen only if the MLN donor rats had been previously immunized with the carrier protein (OA). Further work to investigate whether an active soluble factor was produced by the T cells of Nb-infected rats showed that indeed an "IgE-potentiating factor" was produced. Culture supernatants of T cells from Nb-infected rats selectively enhanced the IgE response of DNP-OA primed cells without affecting the IgG response (61).

It is the selective nature of the potentiating factor in enhancing the formation of IgE but not IgG which distinguishes this factor's activity from that of T cell replacing factor (TRF). TRF activity does not discriminate between antibody responses of different isotypes (62,63) and indeed Suemura and Ishizaka were able to demonstrate a high TRF activity in the supernatants of antigen stimulated MLN cultures. Both IgG and IgE responses were enhanced by the TRF supernatants. More convincing evidence for TRF and the IgE potentiating factor being distinct entities came from fractionation studies using gel filtration. IgE potentiating factor had a relative molecular mass of 10,000 - 20,000 whereas TRF is known to have a relative molecular mass of

about 50,000.

Subsequent work by the Ishizakas and their colleagues on the IgE potentiating factor was directed towards identifying both the source and the target of the factor. It was eventually demonstrated that the target cells of the IgE potentiating factor were B cells. Furthermore, B cells but not T cells were shown to be capable of absorbing the factor.

An important finding in this regard was that the IgE potentiating factor had an affinity for IgE itself; IgE coupled Sepharose absorbed the IgE potentiating factor and activity could be recovered by subsequently passing glycine-HCl buffer, pH 3.0, through the same column (64). Ishizaka and colleagues speculated that it was the affinity for IgE that caused the potentiating factor to focus on IgE bearing B cells and consequently potentiate only the IgE response rather than affecting all isotypes (65). The source of the IgE potentiating factor was conceptually more difficult to accept since it involved the production by T cells, derived from Nb infected rat MLN cells, of an entity which expressed the binding properties of an Fc-IgE receptor (FceR). Notwithstanding this view, Ishizaka and colleagues subsequently demonstrated that although most of the cells which express FceR in normal rats are B cells, following infection with Nb the number of T cells which expressed FceR increased markedly. It was eventually demonstrated that these FceR-bearing T cells were the source of the IgE potentiating factor. The soluble factor expressed,

superficially at least, properties similar to FceR and in fact would inhibit the binding of IgE-coupled SRBC to the FceR(+) T lymphocytes.

Since one of the consequences of Nb infection of rats is an increased level of serum IgE, Ishizaka et al questioned whether IgE itself would affect the FceR(+) T lymphocytes and perhaps cause these cells to release IgE potentiating factor. In a series of in vitro studies these workers showed that addition of rat IgE, at concentrations of 1 μg/ml or higher, to cultures of normal rat lymphocytes did indeed cause an increase in the number of FceR(+) cells from 2-3% of the population to 16%. The effect was restricted to IgE, other immunoglobulin classes such as rat IgG or rabbit IgG failed to increase the number of FceR(+) cells. The addition of rat IgE also did not have any effect on the numbers of Fc receptors with specificity for other immunoglobulin isotypes. Kinetic studies on the appearance of FceR(+) cells showed that the incubation of normal lymphocytes with IgE had to be of 4 - 8 hours duration for the FceR(+) cells to increase in numbers; DNA synthesis was not involved in the expression of detectable FcR with an affinity for IgE (66).

At first sight these studies would seem to point to an uncontrolled production of IgE antibodies following infection with Nb. Any increase in IgE concentration would be expected to lead to the formation of higher numbers of FceR(+) lymphocytes which would in turn release IgE

potentiating factor (IgE binding factor) which would lead to increased production of IgE and the cycle would continue with no obvious controls on the rate of production of IgE. It was from a consideration of the homeostatic mechanisms which might be involved in IgE production that the Ishizakas and their colleagues broadened their search for factors influencing the IgE response to include factors with suppressive activity.

### 1.4.3 IgE suppressive factor

Factors with suppressive activity for the IgE response in fact were readily demonstrated in cultures of MLN from Nb infected rats when the MLN cells were removed 8 days after infection; demonstration of the existence of IgE potentiating factor had been accomplished using MLN cells removed 14 days after infection. The properties of the IgE suppressive factor are similar in several respects to the IgE potentiating factor. Both factors bind to IgE-Sepharose and can be eluted in active form from such columns. Both are produced by MLN cells from Nb infected rats after 24 hours in culture. Both are class specific in their action and they have similar molecular size. The main difference between the two factors is related to their carbohydrate moiety. The potentiating factor binds to lentil lectin-Sepharose and can be eluted from the lectin coupled beads with alpha-methyl-mannoside. In contrast, the IgE specific suppressive factor does not bind to lentil lectin coupled Sepharose beads. Although the initial experiments on the IgE

suppressive factor were conducted with MLN cells removed from rats 8 days after Nb infection subsequent experiments showed that the two IgE specific factors (potentiating and suppressive) were produced over a much wider time period. For example, rat MLN cell cultures established 4 weeks after Nb infection produced, after 24 hours in culture, both the IgE specific potentiating factor and the IgE specific suppressive factor. At this time however, the suppressive factor had a greater activity than the potentiating factor. Thus, it was shown that two factors, both specific for IgE, and with opposite biological function could be produced at different times after parasitic infection with relatively different activities.

A close structural relationship between the IgE potentiating and suppressive factors was suggested by a subsequent report in which the effects of the antibiotic tunicamycin were established (67). Ishizaka et al used a somewhat different approach from previously in that IgE was added to Con-A activated MLN in culture to induce the production of IgE binding factors. Different concentrations of Con A caused different factors to be produced; at 1 μg/ml the MLN cells, on addition of IgE, produced IgE suppressive factor whereas at 10 μg/ml of Con A the MLN produced IgE potentiating factor. A possible relationship between the two factors was suggested by the effects of tunicamycin, an antibiotic which is known to inhibit glycosylation of proteins. In the presence of tunicamycin IgE potentiating

factor was not produced under conditions which otherwise would have led to its production. The production of IgE suppressive factor was not affected by the presence of tunicamycin. The intriguing result was observed when IgE was added in the presence of tunicamycin to MLN cultures which had been activated with 10 μg/ml of Con A. In the absence of tunicamycin, as described above, IgE potentiating factor was not produced but IgE suppressive factor activity was detected.

Ishizaka et al speculated that the IgE potentiating factor and suppressive factor share a common precursor protein and that the biological function of the factors is decided by the process of glycosylation. The authors further speculated that the existence of these two factors, with antagonistic biological functions, could go a long way to explain the selective regulation of the IgE response. An intriguing question in relation to this work is whether a switching mechanism exists for the synthesis of the two factors.

Inhibitory soluble factors affecting selectively the IgE response also have been demonstrated in systems other than those involving parasitic infection. For example, a system was developed by Kishimoto et al (68) in which DNP-derivatized Mycobacterium (DNP-Tbc) was administered to normal mice. These investigators reported that the treatment induced DNP-reactive suppressor T cells capable of exerting selective suppressive effects on antibody responses of the IgE classes. Further work by the same group revealed the ability of the DNP-reactive suppressor T cells to inhibit in a selective

manner IgE antibody responses in vitro either when intact cells were added to such cultures or when soluble mediators released into culture supernatants of DNP-Tbc stimulated DNP-reactive suppressor T cells were incorporated into the cultures (69). The soluble factors produced in this way were subsequently analyzed and it was demonstrated that (a) DNP specific stimulation of DNP-reactive T cells was absolutely required for the induction of the suppressive factor, (b) although antigen specific stimulation was required for induction of the factor, its activity was totally antigen non-specific, (c) the suppressive activity was selective for the IgE class, (d) MHC identity between the factor and its target cells was required for suppressive activity and (e) adsorption on Sepharose-4B-anti-MHC columns revealed that the factor bore determinants encoded by genes in the I-region of the H-2 complex.

Perhaps the most convincing evidence that the factor responsible for these effects is derived from T lymphocytes is to be found in the construction of a T cell hybridoma capable of continuously producing the relevant suppressive factor (70). More recent work on the properties of this factor has revealed the presence of binding sites for IgE molecules as well as an H-2 gene product. It is noteworthy that the factor did not show any affinity for either mouse IgM or IgG (71).

### 1.4.4 Suppressive and Enhancing factors of Allergy

In addition to the IgE specific regulatory factors produced following parasitic infection and administration of hapten-mycobacteria conjugates there exist the "factors of allergy" described by Katz and colleagues. Two IgE selective regulatory factors have been identified thus far, one of which is suppressive (the suppressive factor of allergy, or SFA) and the other of which is enhancing (termed enhancing factor of allergy, or EFA). The respective activities of SFA and EFA have been demonstrated by their abilities to suppress or enhance IgE antibody synthesis in intact mice.

The existence of SFA was first noted in the serum of low IgE responder SJL mice that had been inoculated with Freund's adjuvant containing mycobacteria one week prior to bleeding. When such serum was passively transferred to recipient SJL mice that had been exposed to low dose irradiation in order to convert their IgE phenotype from low responder to high responder, the passively transferred serum completely reversed such IgE responses to the normal low levels. Similar SFA activity could be demonstrated in irradiation - enhanced primary IgE responses and in adoptive secondary responses obtained following passive transfer of primed spleen cells to syngeneic, irradiated low responder recipients (72,73). Studies on the biological properties of SFA have revealed a number of points: (a) SFA is present in varying amounts in the serum of normal mice and the level increases following injection of Freund's complete adjuvant

(FCA), (b) the suppressive effect is selective for IgE antibody responses and is quite anigen non-specific, and (c) it is reported to cross strain and even species barriers. A number of physico-chemical properties of the SFA have also been determined. For example it is stable at 56°C for 60 minutes, is precipitable by ammonium sulphate, has a molecular weight of 150,000 - 200,000 daltons, does not express MHC determinants but does appear to express some determinants identical or cross-reactive with those found on beta-2-microglobulin. As with the IgE specific factors described by Ishizaka et al, for which potentiating and suppressive varieties exist, the counterpart to SFA is EFA. The serum level of EFA, according to Katz, rises following injection of Freund's complete adjuvant and is found in both high and low responder mice. Katz et al report (74) that it is the net balance of SFA versus EFA in a given individual at any given time that determines the phenotype of IgE antibody production following antigen sensitization. In the experimental models described by Katz and colleagues (75), SFA and EFA differ in the kinetics of their appearance following inoculation with FCA. EFA production tends to rise very early after inoculation of FCA, peaking by day 3 or 4 and then subsiding; at the time that EFA subsides, SFA production begins to rise reaching a peak on days 7 - 10. Again there is a similarity between the factors of allergy described by Katz et al on the one hand and the potentiating and suppressive factors described by Ishizaka on the other.

Namely that EFA can be separated from SFA by affinity on Sepharose-Con A columns. EFA has an affinity for Con A greater than SFA, hence the two factors can be separated.

### 1.4.5 Comparison of isotype-specific factors

The properties of the various factors that have been shown to exert regulatory effects on IgE antibody responses have one striking feature in common, namely, their biological effects are restricted to synthesis of IgE antibody molecules.

A second common feature is their ability to act in an antigen non-specific manner, irrespective of whether specific antigenic stimulation might be required to elicit their production. One apparent difference between the IgE potentiating and suppressive (binding) factors on the one hand and the "factors of allergy" on the other is that the former pair of factors were demonstrated only in parasite infected animals. The existence of the factors of allergy was demonstrated in normal animals treated with FCA. In an attempt to relate the activities of the two sets of factors Ishizaka questioned whether the formation of IgE binding factors was limited to lymphocytes from parasite infected animals. On considering the data reported by Katz, Ishizaka envisaged that lymphocytes from FCA-treated animals also produce IgE binding factors. Consequently rats were given two or three injections of FCA per week and their MLN cells were cultured in the presence or absence of IgE. The MLN culture supernatants were shown to contain IgE binding

factors. Culture filtrates from FCA treated animals inhibited rosette formation of FceR(+) cells and moreover the inhibiting factor was absorbed with IgE-Sepharose-4B and recovered in acid eluates indicating that an IgE binding factor was responsible for the inhibition of rosette formation. The binding factor was tested for activity on the antibody response and indeed the IgE anti-DNP-OA response was suppressed without the IgG response being affected. As with the Nb infected rat MLN culture supernatants both suppressive and potentiating factors were recovered from the FCA treated cultures. The factors were separated by fractionation on lentil lectin-Sepharose. At the time of testing the suppressive factor had the higher biological activity (65). Subsequent work by Ishizaka et al demonstrated that the IgE suppressive and potentiating factors could be recovered also from the serum of FCA treated animals as well as from cultures of the MLN of FCA treated animals. The demonstration of the production of IgE binding factor in vivo strengthens Ishizaka's argument that these factors might play an important role in IgE isotype-specific regulation (65). Although some similarity is evident between the IgE binding factors (Ishizaka) and the "factors of allergy" (Katz) it is clear that the factors must be distinct entities; the IgE binding factors have relative molecular masses of 10,000 - 20,000, whereas the suppressive factor of allergy has a relative molecular mass of 150,000 - 200,000. Of particular relevance to the work that forms the subject of this thesis, i.e. the

long term IgE antibody response in BDF1 mice, are the studies which have been carried out on factors which regulate the activation and proliferation of T and B cells in an antigen stimulated response.

## 1.5 Cellular cooperation in antibody production

The need for different populations of lymphocytes to cooperate in the production of antibody was first demonstrated by Claman in 1966. Subsequently the work of several groups of investigators led by Davies, Mitchell and Miller established that the antibody producing cells were derived from the bone marrow (B cells) only after collaboration with a population of thymus derived lymphocytes (T cells) (76).

In 1969 Mitchison et al demonstrated that B and T cells responded to distinct determinants on the same molecule. Spleen cells taken from mice immunized with 4-hydroxy- 5-iodo-3-nitrophenyl (NIP) conjugated to ovalbumin produced a secondary anti-NIP response only when the irradiated recipient of the primed cells was challenged with NIP-OA. A heterologous conjugate NIP-bovine albumin did not stimulate antibody production. This carrier effect, i.e. the enhanced anti-hapten antibody response following immunization with a protein-hapten conjugate which was observed if the animal had been previously immunized with the corresponding carrier alone, was demonstrated for IgE antibody production by Ishizaka in 1973 (77).

The first direct demonstration that T lymphocytes

were required for development of the IgE antibody response was made in 1971 by Okumura and Tada (78). In that work it was demonstrated that neonatally thymectomized rats were unable to produce IgE antibody responses on subsequent immunization with a DNP-carrier conjugate. This defect could be reconstituted by supplementation of such neonatally thymectomized rats with normal or with carrier-primed thymocytes (79). Direct demonstration of T and B cell cooperation in the development of IgE antibody production in mice was made first by Hamaoka et al (80) in 1973. Utilizing an adoptive cell transfer system cellular cooperation between carrier specific and hapten specific mouse spleen cells was shown for the IgE response, as well as for IgG antibodies. The carrier specific cells functioning in this system were shown to be T cells by virtue of their expressing the Thy-1 marker (81).

The adoptive transfer system was widely used to identify further the criteria for cell cooperation. Primary among these is the requirement for histocompatibility of the B and T populations (82). It has been demonstrated that, in an irradiated recipient of hapten specific B cells, antibody production occurred only in the presence of syngeneic carrier primed T cells. Histoincompatible B and T cells could not cooperate to form antibody.

A third cell, the macrophage, was also shown to play an essential role in the initiation of antibody production. Rosenthal and associates demonstrated that, in guinea pigs, T cells from a given strain of animal would proliferate in vitro

only when the antigen present was associated with macrophages from animals of the same strain (83). Again, the ability to cooperate was dependent on the identity, in the two cell populations, of cell surface markers determined by the Ia (immune associated) region of the major histocompatibility complex. Macrophages from a second strain of animal, which differed from the first only in Ia antigens expressed, could not effect cellular proliferation. Similar genetic restrictions for cellular cooperation have been demonstrated using inbred strains of mice.

## 1.6 Soluble mediators of cellular cooperation

Initial theories on the mechanism of cellular interaction proposed a relatively passive role for T cells and macrophages. These cells were thought to act by "focussing" or "presenting" antigen to B cells. More recently this concept has been developed to encompass the accumulating information on soluble mediators that have been shown to result from antigenic stimulation. These mediators, generally referred to as lymphokines, are produced by T cells and are recognized by specific membrane bound receptors on target cells.

Through the efforts of several groups of investigators a working hypothesis has been proposed for T cell activation which involves the interaction of at least 3 distinct cell types (84,85). Stimulation by antigen (or lectin) first activates macrophages to produce a soluble

factor, termed lymphocyte activating factor (LAF) or interluekin-1 (IL-1). IL-1 has been shown to be produced by macrophages (in the solid surface-adherent population) from humans (86), mice (87), guinea pig (88) and dogs (89). In contrast, attempts to identify IL-1 release by lymphoid cell lines or freshly isolated malignant and normal lymphoid cells have been unsuccessful. Various stimuli have been reported to induce IL-1 release from macrophages. Bacterial lipopolysaccharide (LPS) is the prototype IL-1 inducer that has been used experimentally. Human monocytes in particular appear to be exquisitely sensitive to LPS, as little as 10 pg/ml can induce IL-1 release and the optimum concentration appears to be between 1 and 10 ng/ml. Foreign antigen also can cause release of IL-1 from macrophages. In addition, antigen induces a set of T cells the T-responsive cells or T helper-precursors, to express specific surface receptors for IL-2. The same antigen, together with the macrophage derived IL-1 induce another set of T cells, the precursors of T producer cells, to differentiate into cells which produce IL-2. Once the circuit of IL-2 production has been completed, with IL-2 acting on the T helper precursors to promote proliferation, the antigen is no longer necessary for T cell proliferation. Binding of IL-2 to the corresponding IL-2 receptors will induce the T cells to proliferate, which will continue for as long as the growth factor is available (90). One issue that has received attention is whether the T effector cells are a distinct population from the T responsive

cells. Studies on athymic nu/nu have shown that the pre-T cells in these mice do respond to IL-2, however these mice do not contain T cells which will produce IL-2 (91). It is conceivable though that one and the same T cell could both produce and respond to IL-2. Homeostatic control of IL-2 release and the T cell response to IL-2 then becomes more difficult to rationalize and it is necessary to invoke some form of negative feedback regulation to shut down IL-2 production once it has reached a certain level. In relation to this question one report has been published claiming to have detected a soluble inhibitor to IL-2 activity. The factor was detected in serum of normal but not of athymic nu/nu mice (92). Another factor found in culture supernatants, as was described earlier in the context of IgE potentiating factor, is T cell replacing factor (TRF). This factor is believed to act on antigen driven B cells to promote their differentiation (93). The two factors IL-2 and TRF can be separated on the basis of physico-chemical properties and hence can be considered as distinct entities. The relative Molecular mass (Mr) of IL-1 appears to be of the order of 15,000 to 20,000, IL-2 on the other hand presents as a more heterogeneous population of molecules. IL-2 from humans, mice and rats have been reported to have an Mr of 15,000 - 20,000. The majority of IL-2 preparations appear to be heterogeneous giving more than one peak of activity on the separation medium. It was suggested that IL-2 is variably sialylated to explain this heterogeneity (85). TRF has been

reported to have an Mr of 40,000 (93).

The specific signals from T cells in the form of soluble helper factors, together with TRF, drive B cells to antibody formation. TRF expresses an antigen non-specific activity. Both IL-1 and IL-2 may be important mediators of B cell responses but B cell specific growth factors may also exist and act on B cells in a manner analogous to the T cell growth factors (94). The combined activities of the lymphokines just described together with antigen lead to clonal expansion of B cells.

At some stage in this process, and with the effects being more apparent for IgE than with other classes of Ig, IgE antibody responses may be stringently controlled by the non-antigenic specific IgE class specific restricted regulatory cells and their factors described earlier.

## 1.7 Regulation of the established antibody response by anti-idiotype

A system for a regulatory network was proposed by Jerne who recognized that antibodies possess a dual character. While an antibody can recognize an antigenic determinant it is itself immunogenic by virtue of its unique idiotypic determinants (95). As B cells proliferate in response to antigen there is a concomitant expansion of populations of idiotypic determinants. According to Jerne's description, an antigen induces the production of an antibody Ab1, characterized by its idiotype Id1. In turn, the latter

stimulates the synthesis of anti-idiotypic antibody Ab2 bearing the idiotype Id2 that can trigger the production of another anti-(anti-idiotypic) antibody and so on. Moreover, Ab1 should recognize within the immune system any idiotype which happens to resemble antigen i.e. an internal image of the antigen. Jerne assumed that such a network is functional, which means that its regulation should account for the various modes of the immune response.

The complexity of the network is enhanced by the fact that B and T lymphocytes and specific T cell factors have been shown to present idiotypic markers which could participate in such a regulatory process. The importance of idiotype networks has been demonstrated in several systems. Injection of anti-idiotype antibody into neonatal or adult BALB/c mice was shown to prime for T helper cell production where the T cells apparently expressed idiotype(+) receptors. The system used was that associated with anti-phosphoryl choline (anti-PC) antibodies in BALB/c mice. Most of such antibodies have an idiotype corresponding to the myeloma T15. The induction of T helper cells by anti-idiotype was demonstrated in vitro by an enhanced response, in the presence of such T cells to hapten conjugated to protein T-15. The postulated mechanism of induction of helper T cells is through suppression of an idiotype bearing suppressor cell (96).

Studies conducted by Sy et al on the family of idiotypes associated with anti-p-azophenyl arsonate (Ar) antibodies of the A/J mouse strain have shown that there are

two types of suppressor cell, designated Ts1 and Ts2 (97). These cells can each suppress delayed-type hypersensitivity (DTH) responses to the Ar hapten. Ts1 cells have idiotypic receptors and are stimulated by i.v. injection of syngeneic cells to which molecules bearing the cross-reactive idiotype are conjugated. Ts1 and Ts2 elaborate suppressor factors, designated TsF1 and TsF2 which were shown to have binding sites similar to those on the T cell receptors, namely idiotypic (antigen-specific) and anti-idiotypic (idioype-specific) respectively. The TsF1 factor has a Mr of 50,000 is antigen specific and is bound by anti-id antibodies directed to serum anti-Ar antibody. In contrast the TsF2 factor is not bound by anti-idiotype but would bind to idiotype positive molecules (id(+) anti-Ar antibody). Either suppressor factor greatly reduced the anti-Ar DTH response. Injection of the id(+) TsF2 factor stimulates the formation of anti-idiotypic suppressor cell, Ts2. Ts2 act late in the response, i.e. at the affector cell (efferent) stage whereas Ts1 act early in the response and presumably affect the inductive phase (afferent) of the DTH response. The regulatory and complementary link between the two is supported by the finding that injection of TsF1 stimulates the formation of Ts2. These findings support the idea of idiotype networks having a functional role in regulation.

Virtually all of the studies which have been reported on the regulatory role in B cell activation of antigen specific, T cell derived factors have been concerned

with IgM and IgG antibody production. Similarly, in the area of idiotype network regulation and the interaction between idiotype(+) and idiotype-specific T cell subsets, the data has been concerned almost exclusively with DTH and contact sensitivity models together with studies on IgM and IgG antibody production. The exceptions to this statement are few. For example a common idiotypic determinant has been shown in antibody responses of the IgE and IgG classes to the synthetic polypeptide antigen L-glutamic acid L-alanine L-tyrosine (GAT) (98). The T15 idiotypic determinant has been shown in serum IgE antibodies specific for phosphoryl choline (PC) (99) and surface Ig of IgE precursor cells (100).

Idiotypic determinants present on IgE molecules plausibly are functionally important in regulating the production of IgE molecules, since suppression of the IgE antibody response to PC can be achieved by isologous anti-T15 antiserum (99). Recently the existence of the T15 idiotypic determinant has also been demonstrated on PC-mycobacterium primed T cells and PC-specific T cell hybridoma cells (101). Likewise, IgE antibody responses to the benzyl penicilloyl residue coupled to carrier proteins can be suppressed by isologous anti-idiotypic antibodies raised against carrier specific antibodies (102), which suggests that regulatory T cells may be influenced through the idiotypic determinants of antigen-specific receptors on T cells. Apart from these few examples data on the role of lymphokines and idiotype networks on IgE regulation are scant.

## 1.8 T cell regulation of IgE antibody production

A larger body of information dealing specifically with the regulation of IgE antibody synthesis was uncovered by experiments designed to investigate the mechanism which served to limit IgE responses in most rats to a transient pattern and to prevent or minimize the development of significant IgE antibody responses in inbred mice of the low responder phenotype. The decisive work in this regard was reported in a series of papers published by Tada et al beginning in 1971. Utilizing the rat model these investigators demonstrated that certain perturbations such as moderate doses of whole body irradiation (103), adult thymectomy and/or splenectomy (78) or administration of various immunosuppressive drugs (79) converted rats from a characteristic transient low level IgE antibody production to patterns of sustained IgE antibody synthesis of substantial magnitude. The finding that such ehanced IgE responses in rats could be terminated by passive transfer of syngeneic thymocytes or spleen cells (78) provided direct evidence that the limited, transient IgE response patterns in normal rats reflected the dominance of a suppressor T cell regulatory mechanism that normally served to minimize antibody production in this antibody class. The consequences of whole body irradiation and immunosuppressive drug therapy, that is conversion of the normal IgE response pattern to one of persistent high magnitude, reflects the reduction or elimination of the suppressor T lymphocytes

regulating IgE antibody production. Coincident with the initiation of the thesis work was the publication of a series of papers by several authors (104-107) who demonstrated that various manipulations selectively increase the magnitude of IgE antibody production also in mice. These manipulations included low dose whole body irradiation, moderate doses of immunosuppressive drugs such as cyclophosphamide and adult thymectomy. The collective implication of all these studies has been that regulation of IgE antibody synthesis is markedly affected by a suppressive "damping" mechanism which normally serves to limit the magnitude of the IgE antibody response following injection of antigen with the appropriate adjuvant.

## 1.9 Choice of a model

As early as 1920 Coca proposed that allergic individuals were characterized by their ability to continuously produce reagin. This postulate has been borne out by the radioallergosorbent test (RAST), which permits the in vitro measurement of specific IgE in the plasma of atopic individuals. It has been found that the production of specific IgE antibody to pollen persists for many months after exposure to the allergen. Moreover, on re-exposure to pollen during the following season, the IgE antibody concentrations are boosted in a characteristic secondary antibody response.

The above outlines the characteristics of IgE production that investigators have sought to reproduce in animal models i.e. the stimulation, by a small amount of

antigen, of specific, persistent and boosterable production of IgE. The search for these models has revealed a complex of interrelated factors which contribute to IgE production. These include the nature and dose of antigen used, the adjuvant selected for immunization, the species of animal chosen and, within a species, the genotype of the individual. Manipulation of these factors in concert with an examination of their effects on the collaboration of B and T cells for the production of IgE has led to the identification of a number of mechanisms that regulate IgE synthesis.

In the mouse, the capacity to develop high titres of persisting IgE antibody and secondary IgE responses is genetically controlled and linked to the H-2 major histocompatibility complex (MHC) (108,109). The MHC linked genetic control of IgE - low versus high responder phenoype is presumably related to the expression of certain I-region genes on antigen presenting cells. It is known that the recognition by T cells of foreign antigen in conjunction with I region gene products leads to T cell activation.

An important observation made by Levine and Vaz (109) was that persistent high titres of IgE antibody in the mouse could be stimulated by extremely low doses of antigen (1 ug or less) administered in a form precipitated on aluminum hydroxide gel (110). Mice primed in this way were able to develop pronounced secondary responses; interestingly mice primed with high doses of the same antigen elicited little or no IgE antibody production although pronounced IgG antibody

titres were induced. The importance of antigen dosage is seen also on examining the effect of varying amounts of carrier antigen on the magnitude of an IgE anti-hapten response (77). It is necessary to use extremely low doses of carrier (e.g. 0.02 - 0.05 μg OA) in order to demonstrate en enhancement of the IgE response. If immunogenic doses of carrier are used (e.g. 1 μg, which is capable of eliciting a primary IgE response) for the first immunization, the subsequent IgE anti-hapten response is suppressed, notwithstanding an enhanced IgE response. The optimum dose of carrier-antigen required for an optimal IgG anti-hapten response was twenty times higher than the corresponding dose for the IgE anti-hapten antibody response.

The particular IgE antibody response which served as the experimental model in this thesis was selected for study because of the background information available, described above, and because of an unusual but characteristic response of the BDF1 mouse to immunization with low doses of OA. In contrast to the transient IgE response seen in rats, this mouse strain maintained a long lasting, strikingly steady, level of circulating IgE antibody for a year or more following immunization. The various approaches which were taken to try to understand the cellular basis for this persistent response form the subject of this thesis.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Animals

For the first year of this investigation, male B6D2F1 (BDF1) mice, 6-8 weeks of age were purchased from North American Laboratory Supplies, Gunton, Manitoba. For the remainder of the study mice of the same age, strain and sex were obtained from Jackson Laboratories, Bar Harbor, Maine.

Female Long Evans rats, weighing 200-300 grams, purchased from North American Laboratory Supplies, were used for passive cutaneous anaphylaxis (PCA) assays.

Young adult, male New Zealand White (NZW) rabbits were purchased from Riemans Fur Ranch, St. Agatha, Ontario and were used for the production of antisera.

All animals were housed in the Central Animal Care Facilities of the Health Sciences Centre under regulated lighting conditions and were fed commerical food pellets from Allied Mills, Chicago, Illinois and water ad libitum.

## 2.2 Reagents

This section contains an alphabetic listing of the materials used in this study. Sources of reagents used only once are identified in the text of the appropriate Methods section. Antisera are described separately in section 3.3.

Amicon Diaflo Ultrafilters, UM-2, PM-10 and XM-100 were purchased from Amicon, Lexington, Massachusetts.

Alpha-Amylase, 2 x crystallized, of bacterial

origin, was purchased from Sigma Chemical Co., St. Louis, Missouri.

Blue Dextran was purchased from Pharmacia Fine Chemicals, Montreal, Quebec.

Bovine Serum Albumin (BSA), fraction (V), was purchased from ICN Pharmaceuticals Inc., Cleveland, Ohio.

Catalase, purified from bovine liver powder was purchased from Sigma Chemical Co., St. Louis, Missouri.

Concanavalin A (Con A) was obtained from Miles-Yeda Ltd., distributed by Miles Laboratories, Elkhart, Indiana.

Chromium Chloride was purchased from Fisher Scientific Co., Toronto, Ontario.

Cyanogen bromide was purchased from Eastman Organic Co., Rochester, New York, and was used to couple proteins to Sepharose beads using the method described by Cuatrecasas (26).

Dextran Sulphate (DS), as a sodium salt, was purchased from Pharmacia Fine Chemicals, Montreal, Quebec.

Dinitrobenzene sulphonic acid (DNP) was purchased from J.T. Baker, Phillipsburg, New Jersey, and was coupled to OA using the method described by Nisonoff (115).

Evans Blue powder was purchased from Matheson, Coleman and Bell, Norwood, Ohio.

Ferritin, 2 x crystallized, from horse spleen was purchased from Miles Laboratories, Elkhart, Indiana.

Fetal calf sera (FCS) with lot numbers R451121, C631107, P874221, C173028, C775120, E07619 and A976616 were

purchased from Grand Island Biological Co., New York. FCS with lot numbers N50605 and M41512 were purchased from Reheis Chemical Co., Armour Pharmaceuticals, Phoenix, Arizona. These latter two lots of FCS did not contribute to the adoptively transferred response and were used when cell preparation techniques called for the use of FCS. Before use all batches of FCS were heated at 56 C for 30 minutes and filtered through an 0.22 $\mu$ Millipore membrane.

Ficoll was purchased from Pharmacia Fine Chemicals, Montreal, Quebec.

Freund's complete adjuvant (FCA) was purchased from Difco Laboratories, Detroit, Michigan.

Globulin fractions from the sera of BDF1 mice, a normal goat and NZW rabbits were prepared by precipitation with sodium sulphate using the method of Kekwick (111). The concentration of globulin in solution was calculated from the absorbance of the material at 280 nm assuming the absorbance of a 1% solution to be 14.

IgG of mouse origin was prepared by gel filtration on a column of Sephadex G-200. The IgG fraction was used as an antigen for the production of a developing antiserum for visualizing IgG plaque forming cells.

Glutaraldehyde as a 25% solution was purchased from Fisher Scientific Co., Toronto, Ontario.

Keyhole limpet hemocyanin, (KLH), A grade, was purchased as a solution in 50% glycerol from Calbiochem-Behring Co., La Jolla, California.

Lipopolysaccharide B (LPS), prepared from E. coli 055:58 was purchased from Difco Laboratories, Detroit, Michigan.

Lysozyme, purified from from chicken egg white and 3 x crystallized, was purchased from Miles Laboratories (Pty) Ltd., South Africa.

Microtiter trays used for hemagglutination assays and for tissue culture were purchased from Grand Island Biological Company, Grand Island, New York.

Millipore membrane filters were purchased from the Millipore Corporation, Bedford, Massachusetts.

Mitomycin C was purchase from Sigma Chemical Co., St. Louis, Missouri. To inhibit mitosis suspensions of spleen cells were incubated with mitomycin C at a concentration of 40 $\mu$g/ml with $5 \times 10^7$ (112).

Myoglobin, purified from sperm whale skeletal muscle was bought from Sigma Chemical Company, St. Louis, Missouri.

NIP, 4-hydroxy 3-iodo, 5-nitrophenyl acetic acid was purchased from Eastman Kodak, Rochester, New York, and was coupled to OA using the method of Brownstone et al (113).

Normal guinea pig serum, used as a source of complement (C), was purchased from North American Laboratory Supplies, Gunton, Manitoba. To reduce the non-specific cytotoxicity of the material the guinea pig serum was absorbed with agarose (L'Industrie Biologique Francaise obtained from Fisher Scientific) as described by Cohen and Schlesinger (114) before being stored at $-70^{\circ}$C until used.

Omnifluor scintillation cocktail was purchased from New England Nuclear Inc., Lachine, Quebec.

Ovalbumin (OA), purified from chicken egg white and 5 x crystallized was purchased from ICN Pharmaceuticals Inc., Cleveland, Ohio.

Ovomucoid, type III-O from chicken egg white was also purchased from ICN Pharmaceuticals.

Pepsin was purchased from Sigma Chemical Co., St. Louis, Missouri.

Phosphate buffered saline (PBS), 0.008 M $Na_2HPO_4$ and 0.002 $KH_2PO_4$ in 0.14 M NaCl, pH 7.4, was prepared using salts purchased from Fisher Scientific Co., Toronto, Ontario.

Phytohemagglutinin (PHA) was purchased from Difco Laboratories, Detroit, Michigan. The activity of the purchased material was not specified by the supplier so that doses of PHA used in experiments were expressed as volumes of the reconstituted material used.

Pokeweed mitogen (PWM) was purchased from Grand Island Biological Co., New York.

Protein A and Protein-A-Sepharose were purchased from Pharmacia Fine Chemicals Ltd., Montreal, Quebec.

Sephacryl, Sephadex and Sepharose gels were all purchased from Pharmacia Fine Chemicals, Montreal, Quebec.

Sheep red blood cells (SRBC) suspended in Alsever's solution were obtained from North American Laboratory Supplies, Gunton, Manitoba. SRBC were washed three times with 20 volumes of PBS before use.

Sodium azide was purchased from Fisher Scientific Co., Toronto, Ontario.

Sodium metrizoate was purchased from Accurate Chemical and Scientific Corporation, Hicksville, New York.

Tissue culture medium 199 and RPMI 1640 were purchased in powder form, from Grand Island Biological Co., New York and prepared according to the supplier's instructions. All media were sterilized by passage through a 0.22 u Millipore filter into autoclaved glass bottles.

$^{3}$H-Thymidine, 2 Ci/Mol, was purchased from Amersham Searle, Oakville, Ontario.

Trypan blue, in powder form, was purchased from Matheson, Coleman and Bell, Norwood, Ohio.

Water used to prepare reagents was distilled, deionized and finally re-distilled in glass.

## 2.3 Immunization and production of antisera

### 2.3.1 IgE antibody

IgE antibody was induced by immunizing mice with antigen adsorbed to freshly precipitated aluminum hydroxide (109). This was prepared by first mixing 1.0 ml of antigen at a concentration of 100 µg/ml with an equal volume of 10% (w/v) $AlK(SO_4)_2 \cdot 12H_2O$ (Fisher Scientific Co., Toronto, Ontario). Two drops of 1% phenol red were added as a pH indicator. The precipitation of $Al(OH)_3$ was initiated by adding, drop by drop, 1.0 N NaOH until the pH was approximately 7.0. The precipitate of $Al(OH)_3$ to which the

protein was bound, was washed twice with PBS and then diluted 50 fold with a suspension of $Al(OH)_3$ in PBS. The diluent $Al(OH)_3$ had been prepared as above but without the addition of antigen to the starting material. The final suspension contained 1 ug of antigen and 1 mg $Al(OH)_3$ per 0.5 ml. Mice were injected intraperitoneally (i.p.) with 0.5 ml of the prepared mixture. To increase the dose of antigen injected the final dilution with $Al(OH)_3$ was reduced as required.

Ovalbumin, KLH, ferritin, ribonuclease, alpha-amylase, lysozyme, ovomucoid, DNP-OA and NIP-OA were prepared in this way for injection into mice to elicit an IgE antibody response.

To obtain serum samples for assay, mice were warmed by heat lamps to induce dilatation of tail veins. After placing an animal in a restrainer a lateral tail vein was nicked with a scalpel blade. Blood was collected into clean glass test tubes. When pooled serum was to be used as the assay sample an equal volume of blood from all mice within a given group was collected in a single tube. Tubes were placed in a $37^{o}$C water bath for 1 hour, the clots were removed with a wooden applicator stick and allowed to retract completely for 2 - 3 hours in a refrigerator. Serum was obtained by centrifuging clotted blood at 400g for 10 minutes.

Sera to be tested for IgE antibody activity were stored at $-20^{o}$C.

2.3.2 IgG anti-OA

Mouse anti-OA was produced by bi-weekly sub-cutaneous injection of BDF1 mice with 10 μg emulsified in Freund's complete adjuvant. To increase the yield of mouse anti-OA, animals which had been immunized sub-cutaneously were challenged with an i.p. injection of 0.3 ml of emulsion containing 20 μg OA in FCA. Within 3 to 4 weeks after the i.p. injection the majority of animals produced ascitic fluid which was drained by inserting into the abdomen a 25 gauge needle attached to a 3.0 ml syringe. The presence of anti-OA in sera and ascitic fluid was ascertained by precipitate formation in a double diffusion agar plate. In addition the titre of hemagglutinating antibody was determined by monitoring agglutination of OA-coupled SRBC (Section 2.4.2).

Rabbit anti-OA was produced by injecting, at two week intervals, two NZW rabbits each with 1.0 mg of OA emulsified FCA into 4 subcutaneous sites.

Rabbit anti-OA production was monitored in the same manner as was mouse anti-OA. After two injections a strong precipitin line was seen on Ouchterlony analysis. Rabbits were bled from the lateral ear veins at 3 day intervals for two weeks and then sacrificed. Sera were treated in the same manner as described above for mouse sera. Sera to be used in transfer experiments were heated at 56$^{o}$C for 30 minutes before being frozen. The globulin fractions of these antisera were prepared by sodium sulphate precipitation was described in section 2.2.

2.3.3 Cytotoxic antisera

Rabbit antisera to mouse immunoglobulin (RAMIg) was used in a cytotoxic preparative procedure to eliminate B cells from suspensions of lymphocytes. NZW rabbits were injected with 500 ug of mouse gamma globulin in FCA into multiple subcutaneous sites on the back. On testing by immunoelectrophoresis (115) this antiserum showed strong precipitin bands against IgG and IgA and a less pronounced band against IgM. Globulin fractions of RAMIg were prepared either by sodium sulphate precipitation or by passage of whole serum through a column of Protein A-Sepharose using the procedure described by Goding et al (116). This antiserum was also used in a rosette assay for the detection of surface immunoglobulin on B cells.

Rabbit antiserum to mouse T cells was prepared by immunizing NZW rabbits with whole BDF1 mouse brain in FCA as described by Golub (117). Three weeks later the rabbits were challenged with a crude membrane extract of BDF1 thymocytes (118). The resulting antiserum was adsorbed with mouse liver powder (Sigma Chemical Company, St. Louis, Missouri), mouse red blood cells and with syngeneic bone marrow cells. The specificity of the antiserum was assessed by cytotoxicity assay and by measuring the in vitro responsiveness of the surviving cells to Con A and LPS using culture conditions described in section 2.4.3. Mouse anti-Thy 1.2 in the form of IgM monoclonal F7D5 was purchased from Olac Ltd., Blackthorn, Bicester, U.K. Sera to be used in cytotoxicity

assays were heated to 56$^{o}$C for 30 minutes before being frozen.

## 2.4 Assays

### 2.4.1 Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis (PCA) assays were used to determine the IgE antibody titre of mouse serum samples. The general method used was that of Braga and Mota (119). The shaved skin on the back of a Long Evans rat was sensitized by intradermal injections of 50 µl of test serum. For every test sample serial, doubling dilutions of the sample were injected into each of two rats. Dilutions in PBS ranged from 1/2 to 1/8192. Twenty four hours later the rats were injected intravenously with 1.0 ml of PBS containing 1.0 mg of the appropriate antigen and 0.5% (w/v) Evan's blue dye. After twenty minutes the rats were killed and the skin removed from their backs. The PCA titre of a serum sample was defined as the reciprocal of the highest dilution which elicited a blue spot of 5 mm mean diameter on the everted skin surface.

### 2.4.2 Passive hemagglutination

Hemagglutinating antibody (HA) assays were carried out in microtitre trays. Ovalbumin was coupled to washed SRBC using glutaraldehyde (120). Specifically, 2.4 ml of a 2.5% solution of glutaraldehyde were added to 20 ml of a 0.2% (w/v) OA solution in which was suspended 0.8 ml of packed, washed SRBC. After mixing for one hour at room temperature

antigen-coupled cells were washed three times and resuspended in PBS for use. Dilutions of positive and negative control samples were included in each tray used during an assay.

The HA titre of a serum was defined as the reciprocal of the highest dilution of test sample which caused agglutination. To assess the contribution of IgM antibody to the hemagglutinating anti-OA activity aliquots of sera were treated with 0.2 M 2-mercaptoethanol (Fisher Scientific Company, Toronto, Ontario) at room temperature for 60 minutes before assay.

2.4.3 Cytotoxicity testing

Cytotoxicity assays, using antisera directed to the Thy 1.2 antigen or to mouse immunoglobulins, were employed to determine the number of T and B cells respectively in lymphoid cell suspensions.

Generally, the method of Gorer and O'Gorman (121) was used. Briefly, equal volumes of suspended cells at a concentration of $1 \times 10^7$/ml and of optimally diluted antibody were mixed together and held in an ice water bath for 15 minutes. To each assay tube was then added a third volume of diluted, agarose-absorbed normal guinea pig serum as a source of complement. The tubes were then transferred to a $37^{\circ}$C water bath and left for 45 minutes. Viability was determined by the exclusion of Trypan blue dye at a final concentration of 0.1% .

A modification of this technique, suggested by a

report of Yuan, Vitetta and Kettman (122) was introduced into the B cell cytotoxicity assay. This consisted of carrying out all steps of the assay in the presence of 0.01 M sodium azide.

To assess the specificity of the anti-Thy 1 antiserum, the surviving cells were tested in vitro for their responsiveness to Con A and LPS. Cultures were set up in microtitre trays with $6 \times 10^5$ viable cells/culture in RPMI 1640 containing 5% FCS. Cultures were supplemented with medium alone, with LPS at 50 μg/ml or with Con A at 5 μg/ml. It had been previously established that these concentrations of mitogen were optimal for the conditions used. 3-H-Thymidine was added for the final 18 hours of a 72 hours culture. The cells were harvested using a Skatron automatic harvester and were counted in a beta scintillation counter for thymidine incorporation.

### 2.4.4 Rosetting assay for B cells

A rosetting procedure developed in this laboratory by Dr. Robert Schwenk was used as an alternative method of identifying B cells. The method is based on the principle that Ig(+) mouse lymphocytes coated with rabbit anti-mouse Ig will bind, via the Fc region of the rabbit antibody, to Protein A which is coupled to SRBC. Protein A was coupled to red cells by adding 0.5 ml of a stock solution of Protein A (1 mg/ml in 0.9% NaCl) to 1.0 ml of washed SRBC ($1 \times 10^{10}$/ml). With constant mixing, 10 ml of chromium chloride (0.002% in 0.9% NaCl) were then added (123). The cell suspension was kept at 37°C for 45 minutes, washed once with PBS and once

with tissue culture medium before finally being resuspended in 1.0 ml of medium.

Mouse lymphocytes at a concentration of $4 \times 10^7$/ml were mixed with rabbit anti-mouse Ig for 30 minutes at $4^{\circ}$C. During this time the antibody, which had been purified from whole serum by adsorption to a Protein A-Sepharose column, would bind to lymphocytes with available surface immunoglobulin. Cells were washed once in cold tissue culture medium before being resuspended to their starting concentration.

Rosetting was induced by adding 0.2 ml of the RAMIg-coated lymphocytes to 0.2 ml of Protein A coupled SRBC ($2.0 \times 10^9$/ml) and centrifuging the mixture at 500 rpm (IEC model HN) for 5 minutes. The assay tubes were left at room temperature, without disturbance, for a further 30 minutes. The pellet of cells was then thoroughly resuspended in the supernatant using a vortex mixer. An aliquot of the suspension was mixed with a solution of 2% gentian violet in dilute glutaraldehyde (1% in PBS) which fixed the rosettes and also stained the central lymphocyte blue. Rosettes were counted under a microscope using a Spencer-Neubauer hemocytometer and their number was expressed as a percentage of the total number of lymphocytes counted.

Controls for this assay always included the testing of unfractionated spleen cells in which approximately 50% of lymphocytes formed a rosette of red cells. As negative controls RAMIg-coated lymphocytes were mixed with red cells

which had not been reacted with Protein A. A separate tube contained uncoated lymphocytes mixed with Protein A coated SRBC. Earlier experiments had established that thymocytes did not form rosettes when tested in this way.

2.4.5 Plaque forming cell test

Plaque forming cells (PFC) were monitored in experiments designed to assess the effectiveness of the methods used to deplete B or T cells from suspensions of spleen cells.

The PFC assay used was a modification of that first described by Jerne (124). Male BDF1 mice were immunized by intra-venous injection of $5 \times 10^8$ washed SRBC. Three weeks later spleens were removed and a cell suspension, in medium 199, was prepared. B or T cells were removed from aliquots of the starting suspensions using methods to be described in section 2.5. The ability of these depleted populations to support antibody production, both alone and on recombination with each other, was assessed by injecting them into irradiated (600 Rads) syngeneic recipients. Recipients were immediately challenged with $5 \times 10^8$ washed SRBC. Six days later their spleens were removed and their content of plaque forming cells was determined.

Both direct (IgM) and developed (IgG) plaques were measured. The developing antiserum was produced in rabbits immunized with murine IgG. Normal guinea pig serum was used as a complement source to induce red cell lysis at sites of

antibody secretion.

### 2.4.6 Mitogenicity of FCS in vitro

The capacity of six different batches of FCS to stimulate lymphocyte proliferation was tested on spleen cells from normal, 6 to 12 week old BDF1 mice. 5 to 10 x $10^5$ spleen cell per well were cultured in microtitre trays in 200 µl of Eagle's minimum essential medium (Earle's salts) containing glutamine (1 mM, penicillin 100 units/ml, streptomycin 100 µg/ml and FCS. Parallel cultures were set up containing either 5% or 10% FCS. Forty eight hours after initiation of the cultures, the cells in each well were pulsed for 12 hours with 0.2 µCi/mM) and harvested onto glass filters using a Skatron multiple harvester. The filters were dried overnight and counted in an omnifluor toluene cocktail.

## 2.5 Preparation of lymphoid cell suspensions

### 2.5.1 Spleen cells

Spleens were removed from mice, at different times after immunization, into cold Medium 199 supplemented with fetal calf serum. Cell suspensions were made by gently grinding the organs in a wide bore glass homogenizer with a loose fitting teflon plunger. Tissue debris was allowed to sediment for 10 minutes before removing the supernatant cell suspension, passing it through a stainless steel wire mesh sieve (200 mesh) and washing it once, in medium, before use. In certain experiments as required by the protocol primed

spleen cell suspensions were prepared in medium 199 without FCS.

Lymphocytes were counted in a hemocytometer using 1% glacial acetic acid as diluent. Viability was determined by exclusion of Trypan blue dye.

2.5.2 B cells

Isolation of B cells was accomplished by treating suspensions of spleen cells with either rabbit anti-mouse brain associated Thy 1 plus complement or with the monoclonal antibody clone F7D5 anti-Thy 1.2 plus complement using the same conditions, but larger volumes, that were used for the cytotoxicity assay described in section 2.4.3. By using 250 ml glass centrifuge bottles as reaction vessels a spleen cell suspension containing upto $3 \times 10^9$ lymphocytes could be processed at one time. After exposure to cytotoxic antisera dead cells were removed by discontinuous density centrifugation through a solution of Ficoll-Metrizoate (density=1.07) (125). The live cells recovered at the interface of the two phases were washed in tissue culture medium before use.

2.5.3 T cells

T cells were isolated by treating suspensions of spleen cells with rabbit anti-mouse Ig plus complement, again using conditions that were optimal for the cytotoxicity assay used to enumerate cells bearing surface Ig. After exposure

to cytotoxic antisera dead cells were removed in the manner described for B cell preparations.

T cells were also obtained by fractionation of spleen cell suspensions on columns of nylon wool according to the method described by Julius, Simpson and Herzenberg (126). Nylon wool, obtained in the form of Leukopak, LP-1 cartidges (Fenwal Laboratories, Morton Grove, Illinois) was boiled for 15 minutes in 0.01 M EDTA followed by six boilings in distilled water. Disposable plastic syringe barrels were then packed with 600 mg each of the treated, dried nylon wool. Columns were washed with medium 199-5% FCS and warmed to 37° C immediately before use. Each column was loaded with 2.0 ml of whole spleen cell suspension at a concentration of $5 \times 10^7$ /ml. The columns were incubated at 37°C for 1 hour. Non-adherent cells were eluted from the columns with pre-warmed (37°C) medium 199-5% FCS. The recovered, putative T cells were washed once before use in adoptive transfer experiments. For larger scale experiments fractionation was carried out using 50 ml syringes packed with 3 gm dry nylon wool. For these columns, the cell load was proportionately increased to $5 \times 10^8$ in 10 ml.

The percentage of B and T cells in the different cell suspensions prepared was assessed by assaying for the presence of cell surface Ig or Thy 1.2 using the cytotoxicity and rosetting assays described in sections 2.4.3 and 2.4.4. In addition, the capacity of the purified cell populations to contribute to the generation of PFC responses in irradiated

recipients was determined.

2.5.4 Depletion of macrophages

Macrophages were removed from suspensions of spleen cells by passage through a column of Sephadex G-10 and glass beads as described by Ly and Mishell (127) or by layering onto glass Petri dishes. Both procedures were carried out at 37°C and depend on the ability of macrophages to adhere to suitable surfaces. A third method, based on centrifugation through a discontinuous density gradient of Ficoll and sodium metrizoate was also used (128). In the latter procedure $1 \times 10^8$ spleen cells in 5.0 ml medium were layered over 5 ml of a solution of Ficoll-Metrizoate (density = 1.077) and then centrifuged at room temperature for 20 minutes at 400 g. On staining with Wright's stain the cells recovered from the interface were found to be more than 99% small lymphocytes. The majority of the non-lymphoid cells had eosinophil-like characteristics. Approximately 1 in 1000 of the recovered cells had the nucleus and cytoplasmic morphology of macrophages whereas 2% - 3% of the starting cell population appeared to be macrophages.

Such putatively macrophage-depleted cell suspensions were used in adoptive transfer experiments to assess the role of macrophages in the experimental model under investigation.

2.5.5 Manipulation of cells before transfer

As required by some experimental protocols donor cells were subjected to a number of treatments before transfer

to irradiated recipients.

In different experiments donor cells which had been prepared in medium 199 alone were exposed to FCS, antigen, antibody, mitogens or mitomycin C for brief periods before again being washed in medium 199 immediately before adoptive transfer. In all cases aliquots of the cell suspension not exposed to any of the above reagents were incubated in medium 199 and acted as control suspensions in the adoptive transfer system. In all cases the effect of in vitro exposure to these agents on cell viability was assessed by exclusion of 0.1% trypan blue dye.

## 2.6 Cross reactivity of FCS and anti-OA

### 2.6.1 Neutralization of anti-OA

In order to assess whether FCS contained an OA-like antigen experiments were carried out to try to neutralize anti-OA antibody activity with FCS. Pooled sera were obtained from mice after immunization with 1 μg OA in $Al(OH)_3$ i.p. or from irradiated recipients of primed spleen cells. Aliquots of 0.2ml of anti-OA serum were mixed with increasing quantities of OA, FCS, BSA, LPS or normal mouse serum (NMS). These were incubated at 37° C for 1 hour and at 4°C for 18 hours. Residual anti-OA activity was detected by dilutiing each sample in PBS and testing in PCA and HA assays.

### 2.6.2 Affinity chromatography of FCS

Sepharose-4B was activated by cyanogen bromide and

reacted with the globulin fraction of rabbit anti-OA or of goat anti-Ig as described by Cuatrecasas (129). A control aliquot of Sepharose 4B was quenched with ethanolamine (Fisher Scientific Company, Toronto, Ontario) without exposure to any protein solution. These materials were used to make affinity columns of 10 ml each. To each was applied 3.0 ml of FCS Gibco C631107. The samples remained on the column for 90 minutes at room temperature and non-adsorbed material was eluted with PBS until the absorbance at 280 nm was negligible.

Materials which had adsorbed to the columns were eluted with 0.2 M glycine-HCl buffer pH 2.2. Acid eluates were immediately neutralized on emerging from the column. Both the non-adsorbed and the adsorbed fraction from each column were concentrated to the starting sample volume, 3.0 ml, using Amicon PM-10 membranes. The resulting concentrated fractions were each used to supplement suspensions of OA-primed spleen cells for transfer into irradiated recipients.

## 2.7 Gel filtration of FCS

To determine the molecular weight of the supportive factor in FCS an aliquot of FCS was fractionated on a column of Sephacryl A-5M which had been calibrated with markers of the following relative molecular masses; blue dextran ($2 \times 10^6$), ferritin (520,000), catalase (250,000), IgG (150,000), BSA (69,000) and myoglobin (17,000). Fractions were eluted in PBS, selectively pooled, concentrated using Amicon PM-10 and UM-2 membranes and used to supplement suspensions of OA-primed

spleen cells for adoptive transfer.

## 2.8 Preparation of $F(ab')_2$ of anti-OA

A globulin fraction of rabbit anti-OA was prepared by applying whole serum to a column of Protein A-Sepharose and eluting the globulin with 0.58% (v/v) acetic acid in 0.15 M NaCl. The eluted material was immediately neutralized with 1.0 N NaOH, dialyzed overnight against PBS, concentrated using an Amicon XM-100 membrane and dialyzed against 0.1 M sodium acetate buffer pH 5.0. Pepsin digestion was carried out as described by Nisonoff et al (130). The enzyme was added to a final concentration of 3% of the weight of immunoglobulin in solution (0.6 mg pepsin/20 mg Ig). Digestion was carried out at pH 5.0 for 18 hours at $37^{o}C$ and the reaction was stopped by adjusting the pH to 7.2 with 1.0 N NaOH. The digest was dialyzed against PBS and re-applied to a column of Protein A-Sepharose. The fractions which did not bind to the column but which were able to agglutinate OA-coupled SRBC in a hemagglutination assay were recovered and designated the $F(ab')_2$ fragments of anti-OA.

## 2.9 The adoptive transfer system

Donor mice were immunized with 1 μg OA in $Al(OH)_3$ i.p. Four to 12 weeks later suspensions of their spleen cells were prepared in medium 199, washed and resuspended to a concentration of $1 \times 10^{8}$/ml. Recipient mice which 18 hours earlier had been exposed to 600 rads of whole body irradiation

from a $^{60}$Cobalt source were injected with $5 \times 10^7$ primed spleen cells (0.5ml) into a tail vein. Irradiated recipient mice were not challenged with antigen after cell transfer. At intervals after being injected with primed spleen cells, recipient animals were bled; their sera were pooled and tested for IgE and HA to OA.

The spleens of at least six identically treated donor mice were used to make any one suspension of primed spleen cells for adoptive transfer. Each recipient group of mice within a given experiment was composed of 4 to 6 irradiated animals.

# CHAPTER 3: EXPERIMENTAL RESULTS

## 3.1 Expression of data

Much of the data presented in this thesis are derived from the detection of IgE and HA to OA. The levels of both types of antibody are expressed as titres which are the reciprocal of the highest dilution of serum that yields a positive result in either the PCA assay for IgE or the HA assay for IgM and IgG. Most results are expressed as antibody titres. However, in both assays doubling dilutions of sera were tested for activity. For this reason, it is sometimes convenient to express results as the $\log_2$ of the antibody titre. Moreover, the use of doubling dilutions restricts the quantitative interpretation of results, since it is evident that the minimum detectable difference between two samples is one doubling dilution. This means that, in assessing experimental values, a minimal increment above control value represents a 2-fold (or 100%) increase in antibody titre and a minimal decrement represents a 50% decrease in antibody titre.

A second restriction on the quantitative interpretation of results is imposed by the use of pooled sera as test samples. Pooling of sera from 4 to 6 mice was necessary in order to obtain enough sera to accurately carry out the assays. Serum samples were collected from mice after direct immunization or after adoptive transfer of primed cells, these being the two methods used to generate antibody

responses during the studies described in this thesis. Data were collected to determine the variability of the antibody titres among individual sera from mice in a given experimental group. The arithmetic mean values of IgE anti-OA titres determined for the individual samples were compared to titres obtained by assaying pooled sera taken from mice within the same experimental group but which had not contributed to the individual determinations.

Results are presented in Table 1. The differences between the mean titres of four to six individual samples and the titre of pooled sera from groups of five mice ranged from 0.4 to 1.1 $\log_2$ units which is very close to the minimal difference detectable by the PCA assay. This indicates that the values obtained by assaying pooled sera very closely approximate the values that would be obtained if the mean value from a group of individual mice were used to quantitate experimental data.

## 3.2 The IgE anti-OA response in BDF1 mice

The anti-OA responses elicited by the i.p. immunization of BDF1 mice with 1 μg OA in 1 mg $Al(OH)_3$ are shown in Figure 1. IgE anti-OA was first detectable 8 days after immunization and reached a peak between days 10 and 14. Thereafter, the IgE antibody response persisted at a fairly constant level for 8 months or more. HA titres to OA reached a maximum 1 to 2 weeks later than IgE antibody titres, but the responses of these two types of antibody generally paralleled

TABLE 1

A COMPARISON OF IgE ANTI-OA TITRES IN INDIVIDUAL AND POOLED SERA

| Expt. | Days after Immunization | Log IgE anti-OA titre | |
|---|---|---|---|
| | | Individual Sera | Pooled sera (b*) |
| | | Mean ± S.D. (n) | (n=5) |
| A | 9 | 7.8 ± 1.9 (5) | 7.4 |
| B | 10 | 10.7 ± 0.9 (6) | 11.7 |
| C | 14 | 10.1 ± 1.9 (5) | 11.2 |
| D | 14 | 9.4 ± 1.3 (4) | 8.7 |
| | Days after Transfer (c*) | | |
| E | 8 | 8.8 ± 0.5 (4) | 9.3 |
| F | 13 | 8.5 ± 0.7 (4) | 9.0 |
| G | 31 | 8.9 ± 0.7 (4) | 9.4 |

a*
Sera were obtained after immunization with 1 μg OA in $Al(OH)_3$ i.p.

b*
Pooled sera were obtained from mice other than those which had been bled for individual samples.

c*
Sera were obtained from irradiated recipients at different times after injection of $5 \times 10^7$ OA-primed cells i.v.

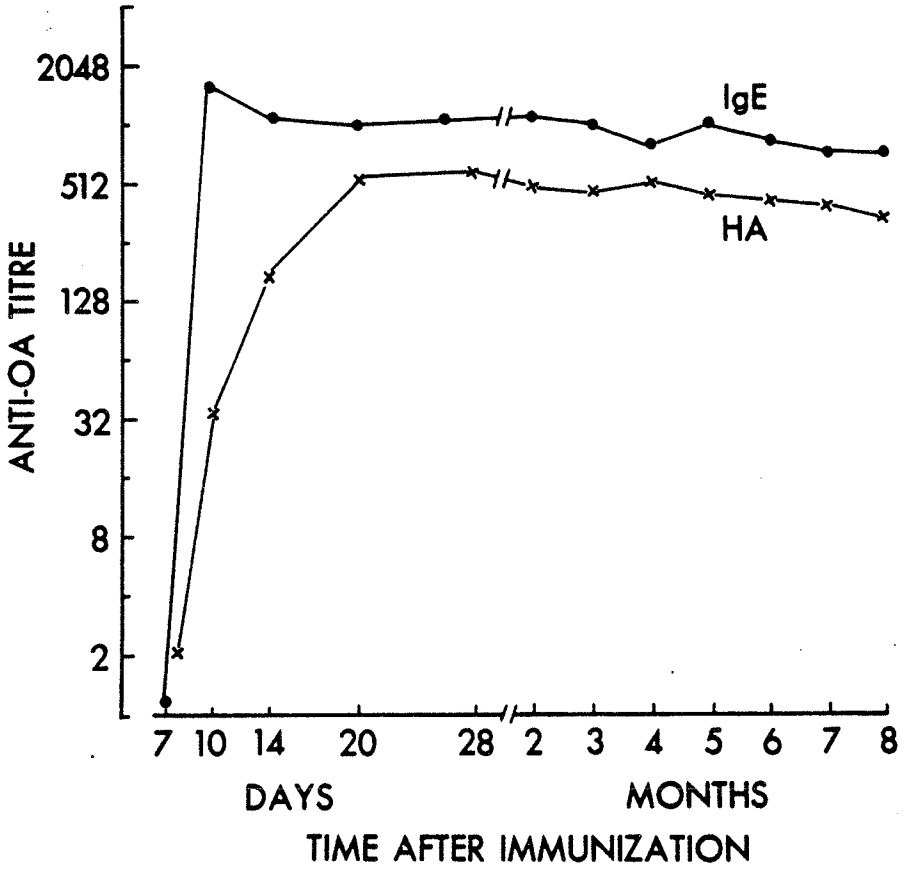


Figure 1.

The anti-OA titres stimulated by the i.p. immunization of male BDF1 mice with 1 μg OA in 1 mg $Al(OH)_3$. IgE anti-OA titres (•) were determined by passive cutaneous anaphylaxis in rats. The hemagglutinating antibody titres (x) of the same sera were also determined. Titres are recorded as the reciprocal of the highest dilution giving a positive determination.

each other over the long term. Treatment of antisera with 0.1 M 2-mercaptoethanol reduced the HA titre by one doubling dilution, indicating that both IgG and IgM contributed to the HA response. However, given the greater efficiency of IgM antibodies in causing hemagglutination it is probable that this apparent drop of 50% is actually attributable to much less than half of the total antibody content. Therefore it is likely that the major class of antibody detected by hemagglutination is IgG.

The magnitude and persistence of the IgE anti-OA response is in striking contrast to that elicited by six other antigens prepared and tested using the same procedure as for OA. Figure 2 reveals that at doses of 1 μg per mouse only KLH induced a modest IgE antibody response. Ferritin and alpha-amylase induced very transient responses, while ribonuclease, lysozyme and ovomucoid were ineffective immunogens under these conditions.

## 3.3 Half-life of circulating mouse IgE

To assess whether the persistence of the IgE anti-OA response could be attributed to a particularly slow catabolism of this immunoglobulin isotype, the half-life of this antibody in the circulation of mice was determined. This was done by measuring residual IgE anti-OA activity in the sera of mice which had been passively immunized with the antibody. Several groups of mice were immunized to allow for a relatively low frequency of bleedings. Bleedings from any

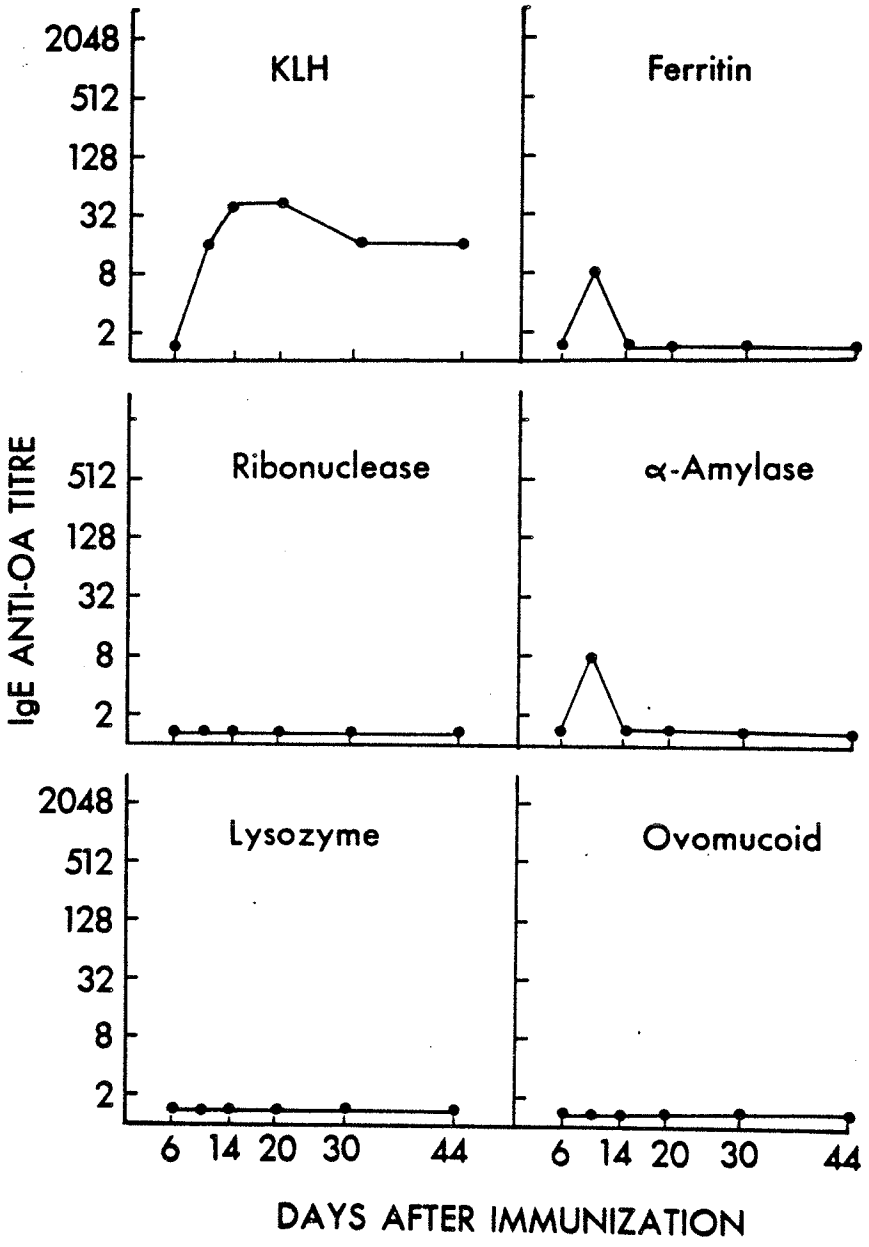


Figure 2.

IgE antibody titres stimulated by immunization of BDF1 mice with KLH, ferritin, ribonuclease, α-amylase, lysozyme or ovomucoid. Each antigen was injected at a dose of 1 μg in 1 mg $Al(OH)_3$ i.p.

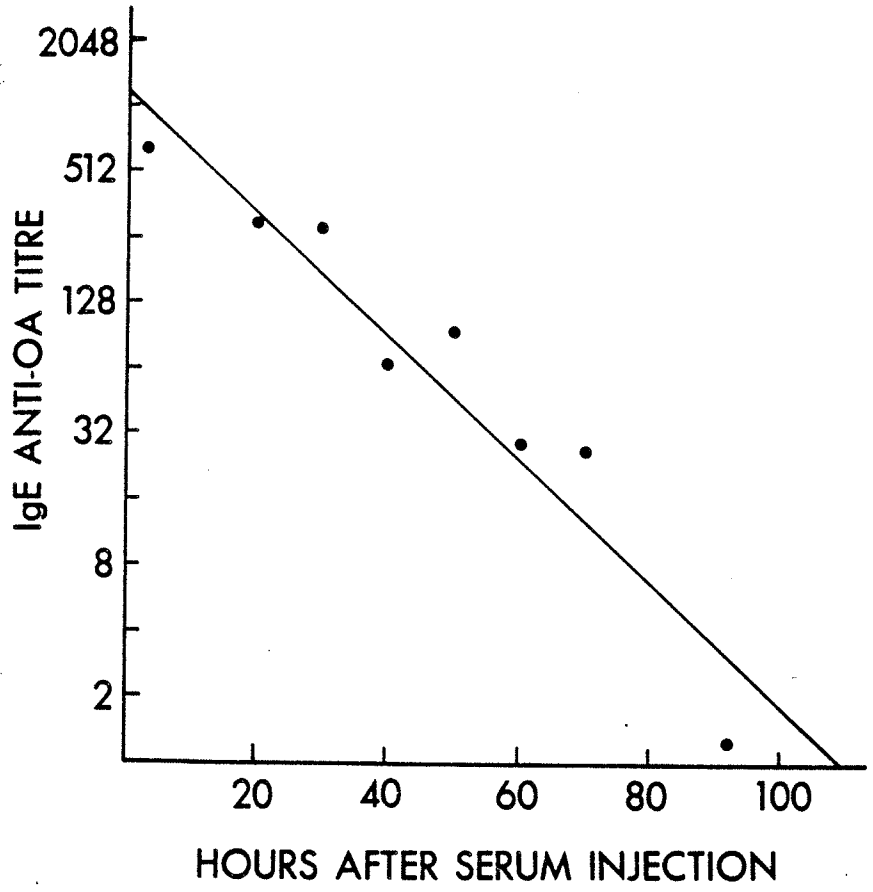


Figure 3.

The elimination of mouse IgE anti-OA from the circulation of normal mice which had been passively immunized with mouse serum containing a high titre of the antibody. Residual antibody was determined by heterologous PCA assay. The straight line was fitted to the data using the method of least squares.

one group were taken at 32 hour intervals. Residual IgE anti-OA was measured by PCA and the resulting titres expressed as a function of time (Figure 3). Regression analysis of the data indicated that the half-life of IgE anti-OA in serum was 10.5 hours. This short half-life obviously argues against the persistence of circulating antibody several months after immunization. Moreover, the half-life of plasma cells producing antibody had been reported to be in the order of a few days (131,132). As a result, it was felt at this stage that continued recruitment of cells to the status of antibody secretors probably underlay the long-term response and a model was sought which would permit examination of the factors that regulated that recruitment.

## 3.4 Antigen independent adoptive transfer of anti-OA production

To study the cell recruitment patterns underlying persistent IgE and IgG antibody production, an adoptive transfer system based on that described by Kontiainen and Mitchison was used (133). These authors had found that spleen cells from mice immunized with fowl gamma globulin, after incubation at 37° C and transfer to irradiated recipients, produced antibody without further antigenic challenge of the recipients. Antibody had been detected by using a Farr-type of immunoassay. Such a model was particularly attractive because it did not require the introduction of exogenous antigen which would have

superimposed a new wave of cellular differentiation on the established immune response.

Following this approach, a cell suspension was prepared from the spleens of mice that had been injected i.p. 28 days earlier with 1 μg of OA in $Al(OH)_3$. $5 \times 10^7$ primed cells suspended in Medium 199 containing 20% FCS were injected into irradiated recipients. Recipients were not challenged with OA after transfer. Figure 4 shows that IgE anti-OA was detectable in the circulation of recipients by day 5 after transfer and that by day 7 the IgE antibody levels approached those of the donor mice at the time of sacrifice. The adoptive IgE anti-OA response persisted for up to 6 months. Hemagglutinating antibody to OA also appeared in the circulation of recipient mice and also reached levels comparable to those found in donor mice.

Unlike the system described by Kontiainen and Mitchison, the production of an antigen-independent anti-OA response depended neither on repeated immunization of donor mice nor on the incubation of primed spleen cells at 37°C before transfer to irradiated recipients. Figure 5 shows that washed, primed spleen cells kept at 4 °C for one hour before transfer were as effective in supporting the adoptive response as were cells incubated at 37°C for the same period of time.

To determine whether the capacity to adoptively transfer anti-OA production, in the absence of antigenic challenge, persisted in the irradiated recipients attempts

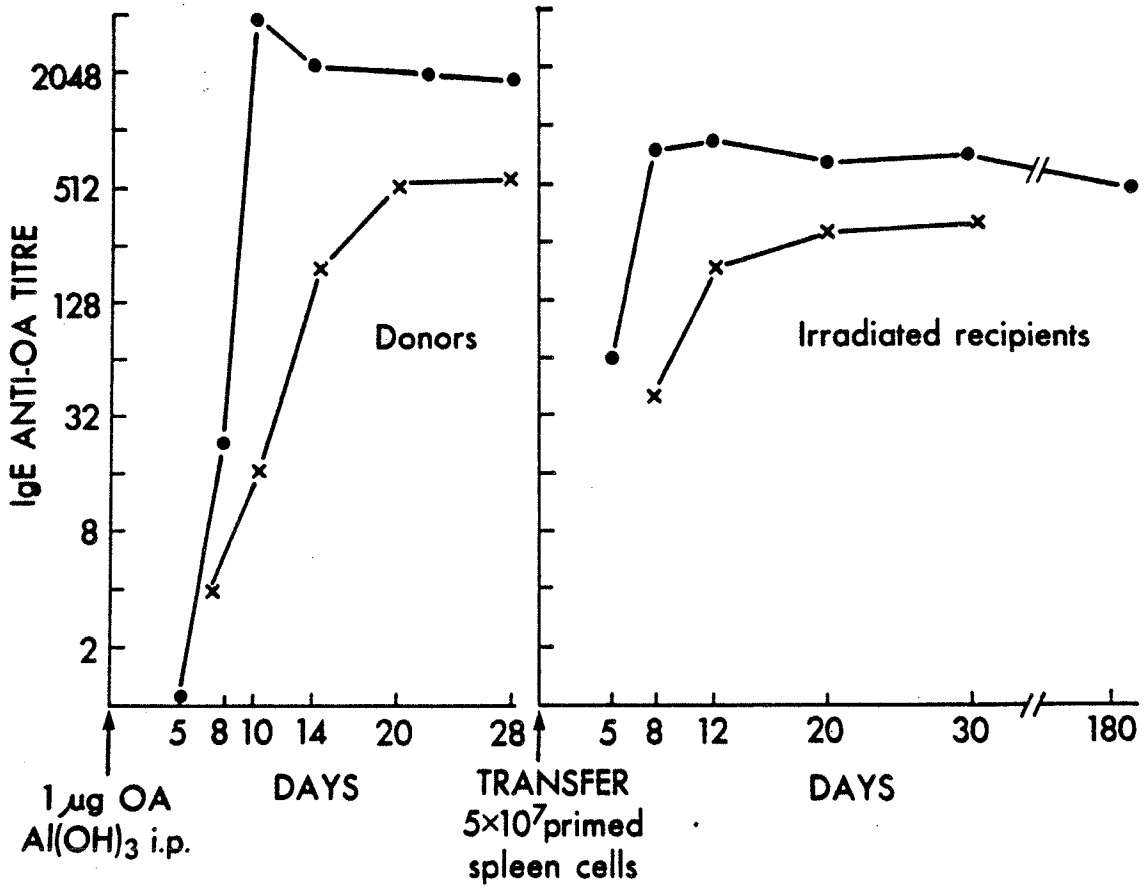


Figure 4

Adoptive transfer of IgE and hemagglutinating anti-OA production without antigenic challenge of the irradiated recipients. OA-primed cells, obtained from the spleens of mice four weeks after immunization, were injected i.v. into syngeneic irradiated (600 Rads) recipients. Each of six recipients received $5 \times 10^7$ primed cells. Irradiated recipients were not challenged with OA. IgE antibody (•) and HA (x) titres to OA were determined in the sera of donors and recipients.

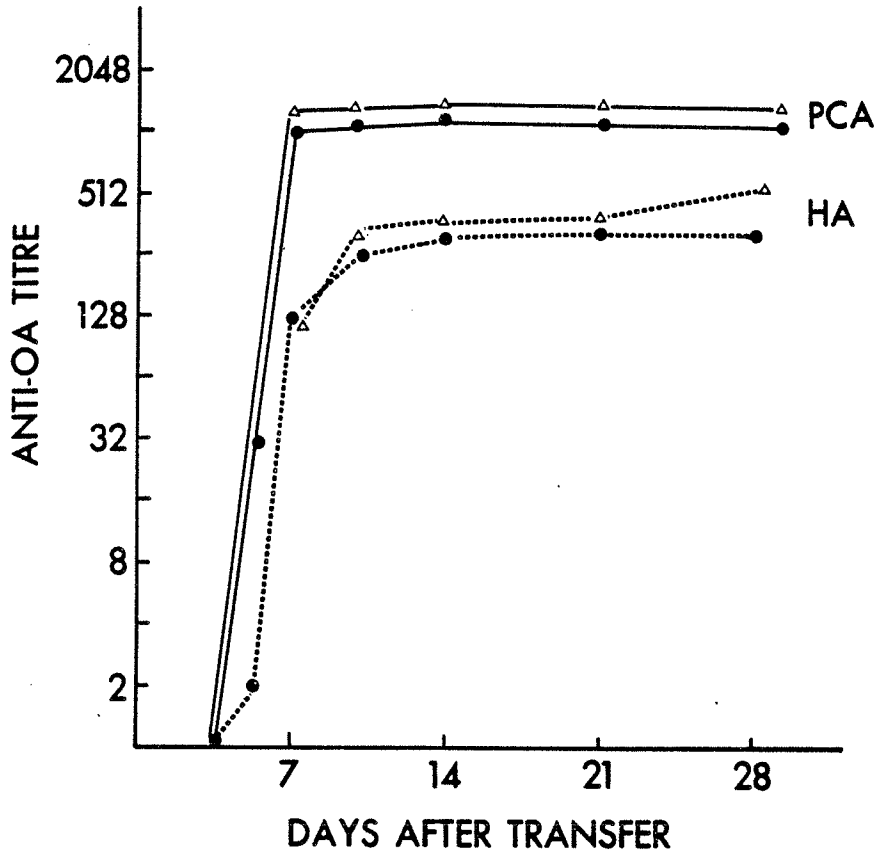


Figure 5.

The effect of incubation on the transfer of anti-OA production to irradiated recipients in the absence of antigenic challenge. Spleen cells from mice immunized four weeks earlier were incubated at 4°C (●) or 37° C (Δ) for 60 min., washed in Medium 199 and injected i.v. into groups of six irradiated mice. Each recipient received 5 x $10^7$ primed cells. The PCA (———) and HA (-------) titres of the pooled sera of recipients were determined.

were made to transfer the antigen independent response to a secondary recipient. Spleens were removed from recipients of OA-primed cells 35 days after the initial transfer. Cell suspensions were prepared in Medium 199-FCS and $5 \times 10^7$ cells were injected into each of the secondary recipients. Figure 6 shows that, in the secondary host, IgE anti-OA could not be detected in the circulation. Hemagglutinating antibody to OA, however, appeared in the serum 5 days after transfer and reached maximal levels 15 days later.

The rise of HA titres in the sera of secondary recipients was slower than had been observed in the primary recipient. However, the maximal HA anti-OA titres reached by the secondary recipient were only slightly lower than seen in the primary recipient.

3.4.1 Transfer of cells primed by antigens other than OA

Since primary IgE antibody responses to antigens other than OA were very weak at doses of 1 μg (Figure 2), higher doses and multiple immunizations were used in an attempt to generate substantial IgE antibody titres to KLH, alpha-amylase and ribonuclease. Doses of 10 μg in 1 mg of $Al(OH)_3$ were effective for alpha-amylase and ribonuclease whereas 100 ug of KLH was necessary to elicit titres greater than 200. For all these antigens, however, multiple immunizations with 1 to 10 μg of antigen in adjuvant generated high and persistent IgE antibody titres. Having produced IgE antibody to these antigens with titres which were comparable

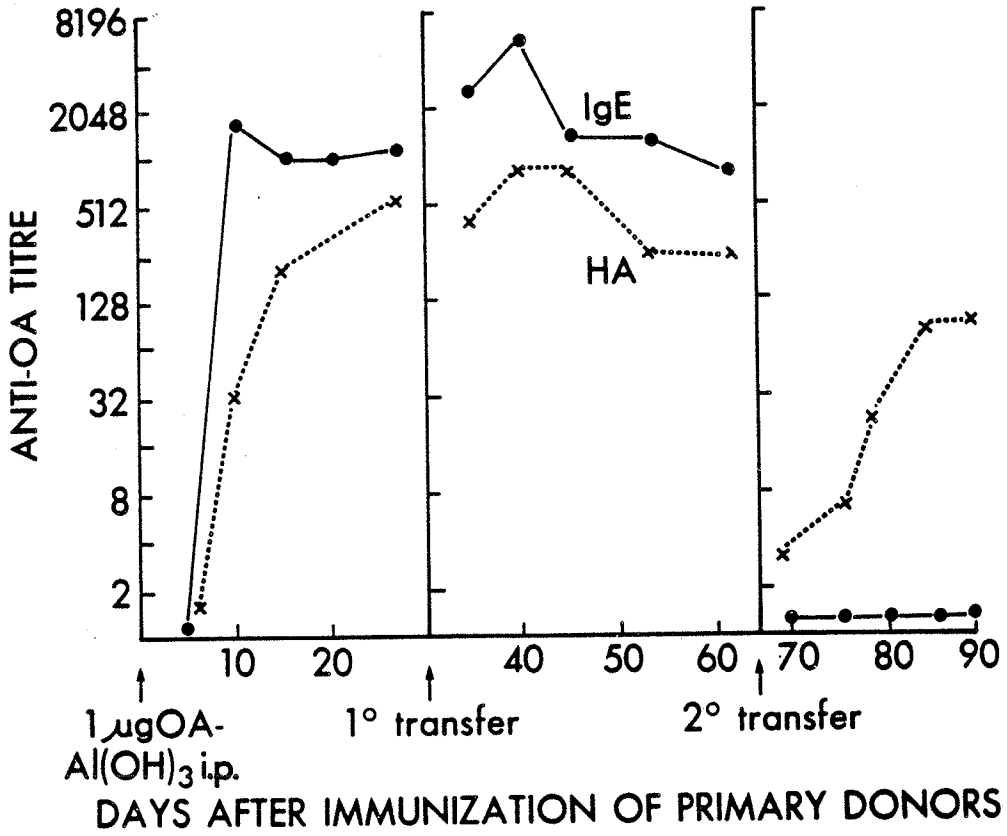


Figure 6.

Secondary adoptive transfer of anti-OA production without antigenic challenge of the irradiated recipients. Thirty days after immunization of donor mice with 1 µg OA in $Al(OH)_3$ i.p., $5 \times 10^7$ primed spleen cells, suspended in Medium 199-20% FCS, were transferred into each of a group of eight irradiated recipients. Thirty-five days later, the spleen cells from the primary recipients were transferred to a second group of irradiated recipients. IgE antibody (•) and HA (×) to OA were monitored in the sera of all three groups of mice.

to those generated by OA, attempts were made to adoptively transfer the immune response to irradiated recipients using primed spleen cells without the corresponding antigenic challenge to the recipients. Figure 7 shows that spleen cells taken from mice which, after three immunizations with alpha-amylase, had high circulating IgE antibody titres, were unable to generate an adoptive IgE anti-alpha-amylase response when transferred without added antigen to irradiated recipients. Figure 8 shows that cells taken from mice immunized with increasing doses of KLH also did not support an antigen independent adoptive anti-KLH response.

Experiments were also carried out in an attempt to transfer an antigen independent anti-hapten response to irradiated recipients. Mice were immunized with 1 ug of $DNP_5$-OA in 1 mg of $Al(OH)_3$ i.p. This resulted in the production of high titres of IgE antibody to both OA and to DNP. Nevertheless, when spleen cells were taken from such primed animals and transferred to irradiated recipients, only an early, transient anti-DNP response could be detected. The IgE anti-OA response, however, was efficiently transferred to these same recipients (Figure 9). When cells primed by immunization with $NIP_{2.2}$-OA were used in an adoptive transfer experiment, similar results were observed. The anti-hapten response was not generated in the recipients while the anti-carrier response was prominent (data not shown).

Despite the inability to reproduce, with other antigens, an antigen independent adoptive transfer it was

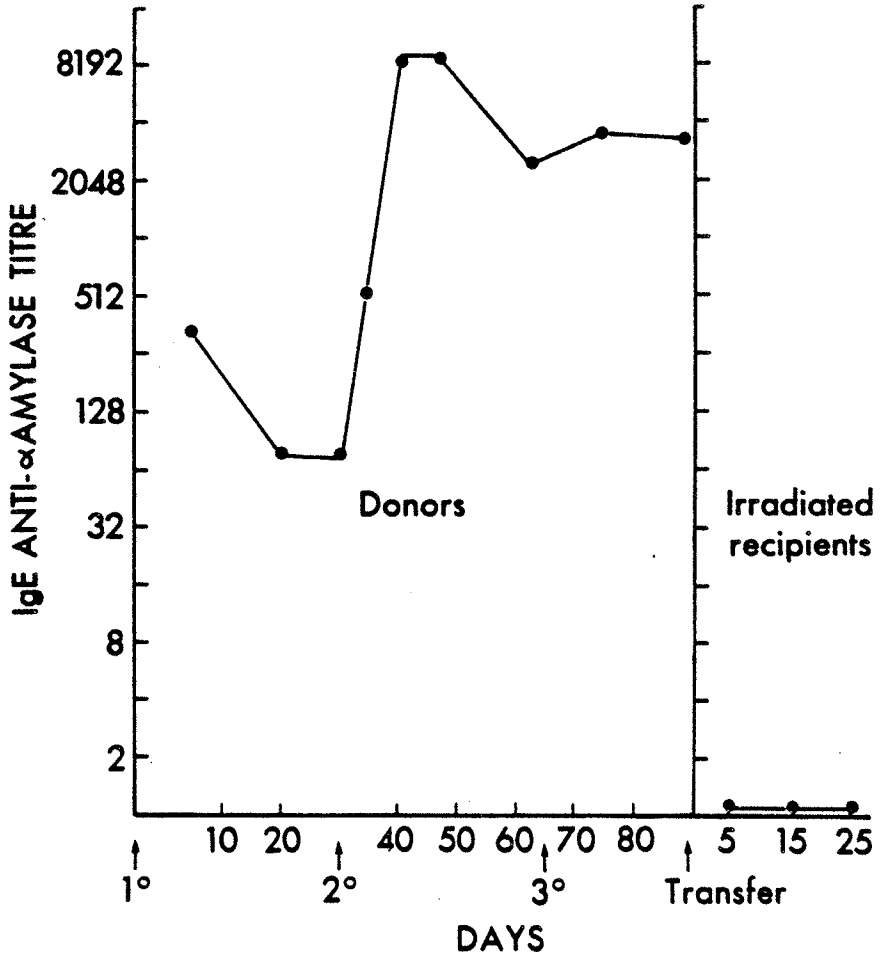


Figure 7.

Transfer of α-amylase primed cells to irradiated recipients. Eight BDF1 mice were immunized with a primary injection of 10 μg α-amylase in 1 mg $Al(OH)_3$ i.p., followed at four-week intervals by secondary and tertiary immunizations with 1 μg of antigen in adjuvant. Twenty-five days after the last injection the mice were sacrificed and their spleen cells were suspended in Medium 199-20% FCS and injected, i.v., into irradiated recipients. Each recipient received $5 \times 10^7$ primed cells. IgE anti-α-amylase was detected by PCA assay.

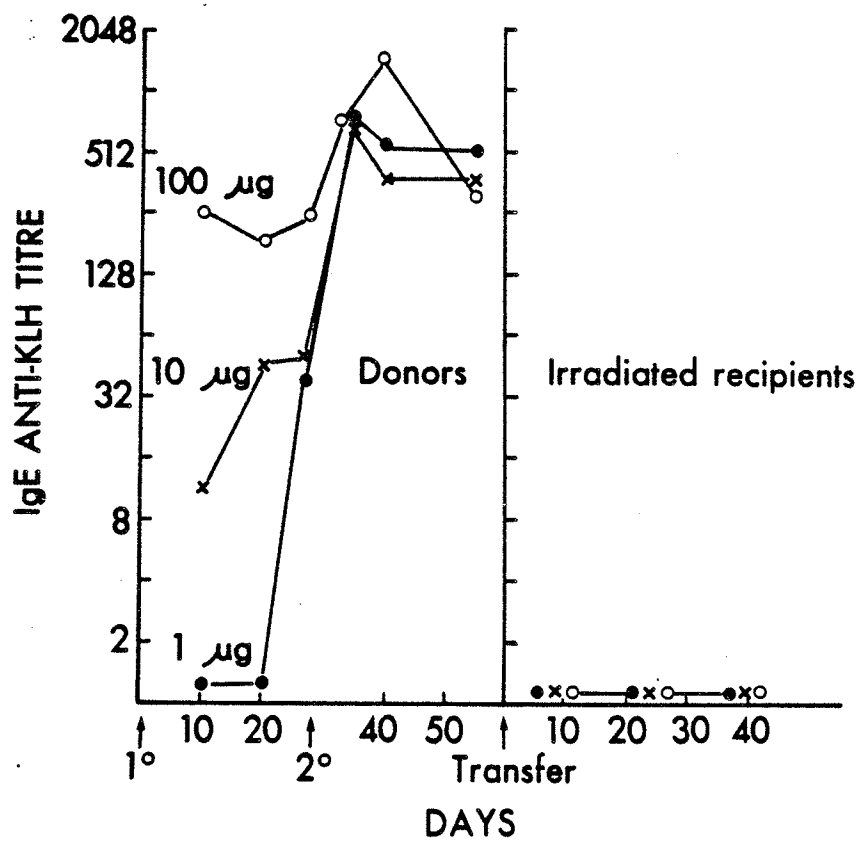


Figure 8.

Transfer of KLH-primed cells to irradiated recipients. Groups of eight BDF1 mice were immunized with 1 µg (•), 10 µg (x) or 100 ug (o) KLH in 1 mg $Al(OH)_3$ i.p. Four weeks later a secondary injection of 1 µg KLH in adjuvant was administered i.p. One month later, suspensions of spleen cells from each of the three groups of immunized mice were prepared in Medium 199-20% FCS and injected into groups of irradiated (600 R) recipients. Each recipient received $5 \times 10^7$ primed cells i.v. IgE anti-KLH was detected by PCA assay.

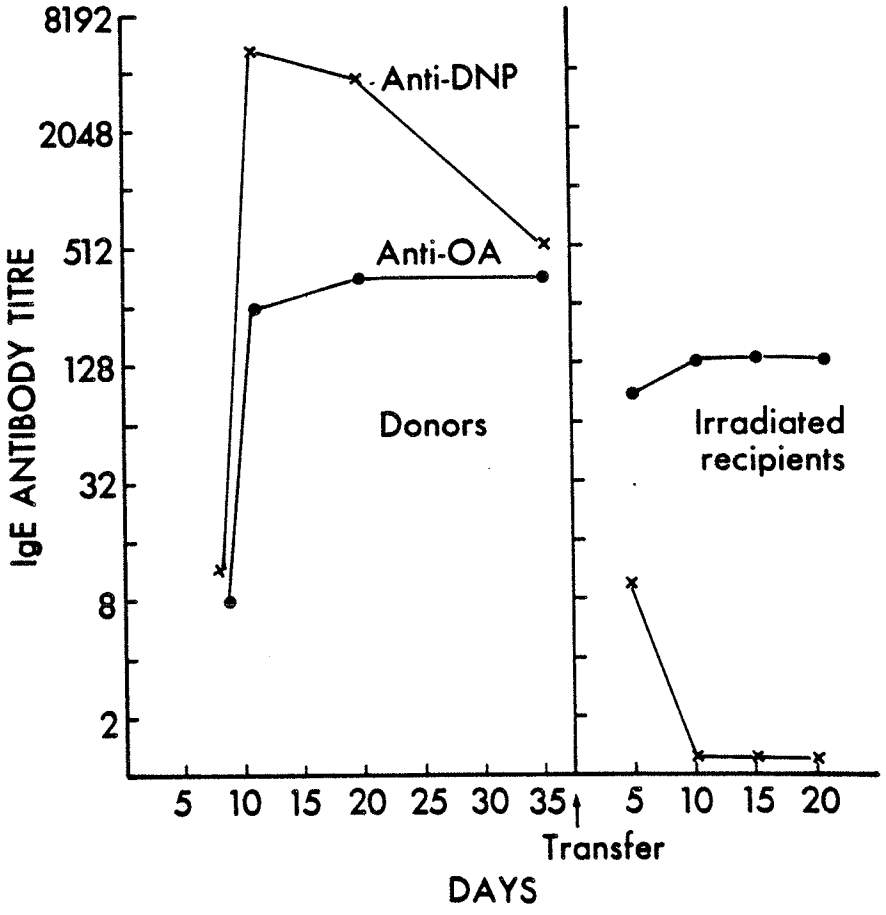


Figure 9.

Transfer of DNP-OA primed cells to irradiated recipients. Eight BDF1 mice were each immunized i.p. with 1 μg DNP -OA in 1 mg $Al(OH)_3$. Five weeks later spleen cells from these mice were suspended in Medium 199-20% FCS and injected i.v. into irradiated recipients. Each recipient received $5 \times 10^7$ primed cells. IgE antibody to DNP (×) and to OA (●) were determined by PCA using DNP-Ficoll or OA to challenge sensitized rats.

decided that the observation of such a phenomenon with OA was interesting enough to warrant further investigation. Experiments were therefore carried out to characterize and analyze the adoptive anti-OA response.

### 3.4.2 Evidence for the donor origin of the adoptive IgE anti-OA response

It was important to establish the origin of the IgE anti-OA antibody detected in the circulation of the irradiated recipients of primed spleen cells. The first experiment in this series was designed to determine whether the irradiated recipients were able to mount an immune response following conventional immunization in the absence of lymphocytic reconstitution. Four groups of irradiated mice were injected with 1 μg OA in $Al(OH)_3$ i.p. at different times after irradiation. Figure 10 shows that irradiated mice failed to produce an IgE anti-OA response when immunized upto 7 days after irradiation. Previous experiments (Figure 4) had shown that by day 7 after irradiation and transfer of primed cells without antigenic challenge to the recipient, the adoptive IgE anti-OA response had already reached its maximal level. This demonstrated that irradiated mice alone were quite unable to generate an immune response within the time required for the adoptively transferred response to manifest itself.

Further evidence that the adoptive anti-OA response was dependent on some property of the donor's cell population was obtained by injecting different groups of irradiated mice

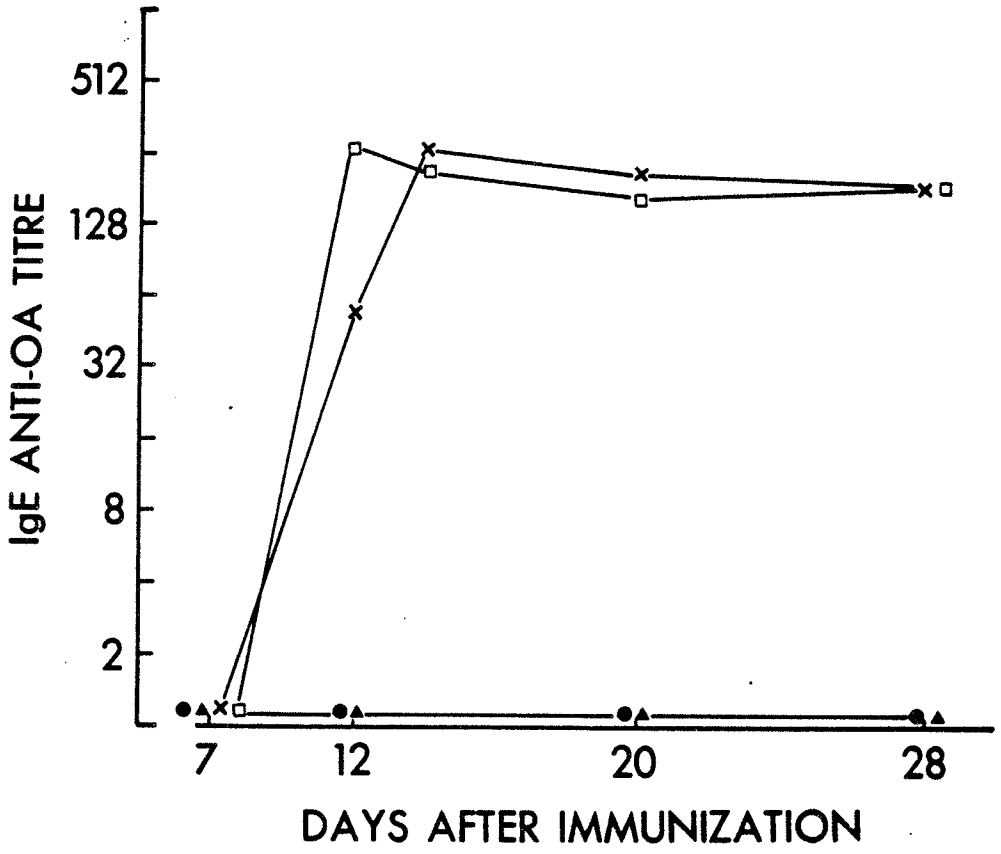


Figure 10.

Immunological incompetence of irradiated recipients. Twenty-four mice were exposed to 600 Rads. Groups of six irradiated mice were immunized i.p. with 1 μg OA in $Al(OH)_3$ 24 hr. (●), 7 days (▲), 14 days (x) or 21 days (□) after irradiation.

with increasing numbers of primed cells. When the sera of the recipients were assayed for IgE anti-OA activity, it was shown that, with the transfer of increasing numbers of cells the maximal level of antibody in the recipient mice increased in a linear fashion and reached a plateau when mice received 1 x $10^8$ primed cells (Figure 11).

Maximal titres of hemagglutinating antibody to OA also rose as the number of cells transferred was increased.

### 3.4.3 Development of donor cell capacity to support the transferred response

Primed spleen cells were transferred to irradiated recipients at various times after intra-peritoneal immunization of donors with 1 μg OA in $Al(OH)_3$. Cells taken from donors 3 or 7 days after immunization did not generate an adoptive IgE anti-OA response in irradiated recipients (Table 2). Very low levels of both IgE and HA were detected in the sera of recipients of cells obtained 10 to 14 days after immunization whereas cells transferred 4 to 8 weeks after immunization produced an optimal response to irradiated recipients. It is noteworthy that maximal levels of IgE anti-OA were measured in the donor mice 10 to 14 days after immunization at which time their spleen cells were capable of supporting only a very weak adoptive response.

The capacity of primed spleen cells, obtained more than 8 weeks after immunization, to produce IgE anti-OA in irradiated recipients was also investigated. The first time

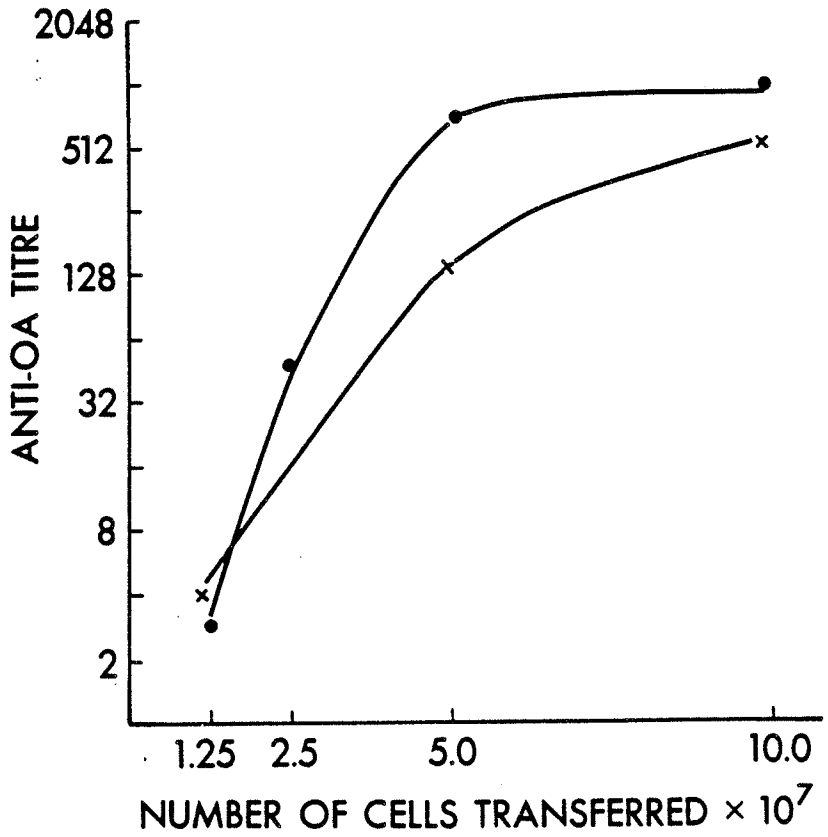


Figure 11.

The effect of transferring increasing numbers of OA-primed spleen cells on the magnitude of the adoptive anti-OA response. Results show the IgE (•) and hemagglutinating (×) anti-OA titres in the sera of recipients ten days after transfer of primed cells.

TABLE 2

THE ABILITY OF PRIMED SPLEEN CELLS TO TRANSFER THE ANTI-OA RESPONSE WITHIN TWO MONTHS AFTER IMMUNIZATION

| Time of transfer Days after immunization (a*) | Titre in donor serum at time of transfer (b*) | Titre in recipient serum, Day 12 after transfer (c*) |
|---|---|---|
| 3 | 0 | 0 |
| 7 | 0 | 0 |
| 10 | 1024 | 8 |
| 14 | 2048 | 32 |
| 28 | 1024 | 512 |
| 56 | 512 | 512 |

a*
Donors were immunized with 1 μg OA in 1 mg $Al(OH)_3$ i.p.

b*
IgE anti-OA titres were determined in sera obtained from donor mice at the time of sacrifice.

c*
Irradiated recipients were injected with $5 \times 10^7$ primed cells suspended in Medium 199 supplemented with 20% FCS (Gibco C631107).

this experiment was conducted it was noted that, beyond 8 weeks after immunization, primed spleen cells had a steadily decreasing capacity to support an antigen independent adoptive response despite the persistence of high levels of anti-OA in the circulation of donor animals (Figure 12). When later investigations revealed that different batches of fetal calf sera had different effects on the magnitude of the transferred response the experiment was repeated with care taken to use a single batch of FCS for the six months of the experiments. On repetition of the experiment the ability of primed spleen cells to support an adoptive response was observed to remain essentially unchanged. Figure 13 illustrates that at 24 weeks after immunization primed spleen cells were capable of supporting a transferred response of a magnitude equivalent to that produced by cells 4 weeks after immunization. In both cases the maximal IgE anti-OA titres reached by the recipients were comparable to those in the donor mice. The inability of primed spleen cells obtained during the first 2 - 3 weeks after immunization to support a substantial adoptive IgE anti-OA response was, however, again observed. Investigations into the possible roles of FCS in the adoptive response will be discussed in section 3.5.

Data from existing experiments were collated to determine whether one could, indeed, expect a correlation of antibody titres in donors and recipients. All experiments in which donor mice had been sacrificed 4 to 8 weeks after immunization with 1 ug OA in $Al(OH)_3$ were included. When the

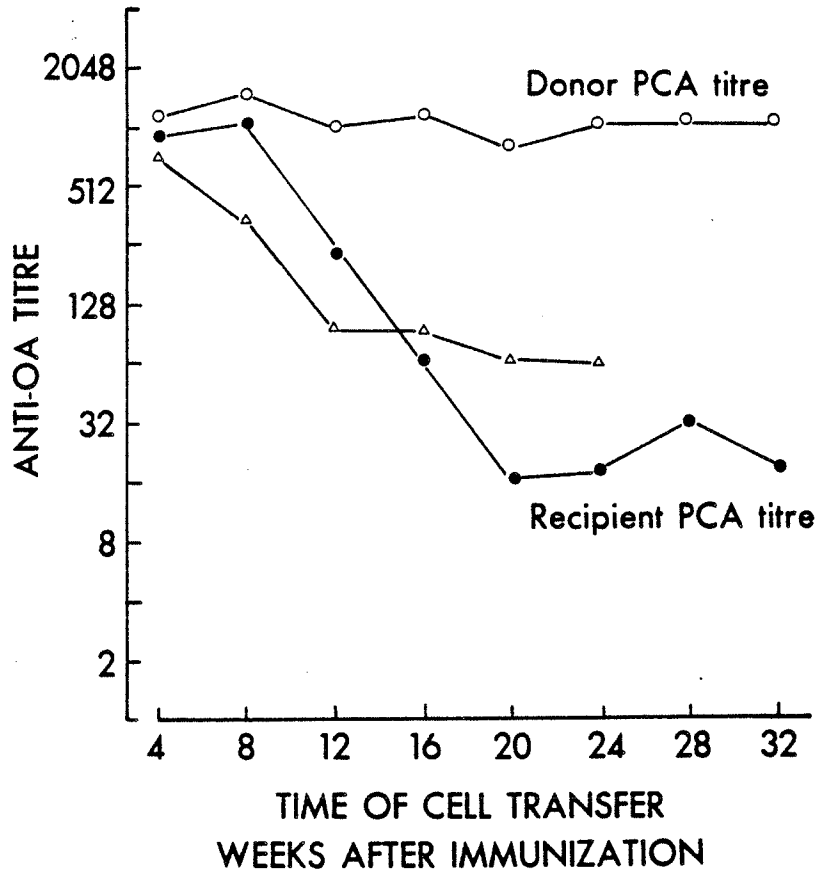


Figure 12.

The effect of time after immunization on the ability of primed spleen cells to transfer anti-OA production to irradiated recipients. Mice were immunized i.p. with 1 μg OA in 1 mg $Al(OH)_3$ . At monthly intervals after immunization, the spleens of six mice were used as a source of OA-primed cells which were suspended in Medium 199-20% FCS and injected into irradiated recipients. The PCA titres (●) in the sera of recipient mice were determined 12 days after cell transfer. Also shown are the PCA titres of donor sera at the time of transfer (o).

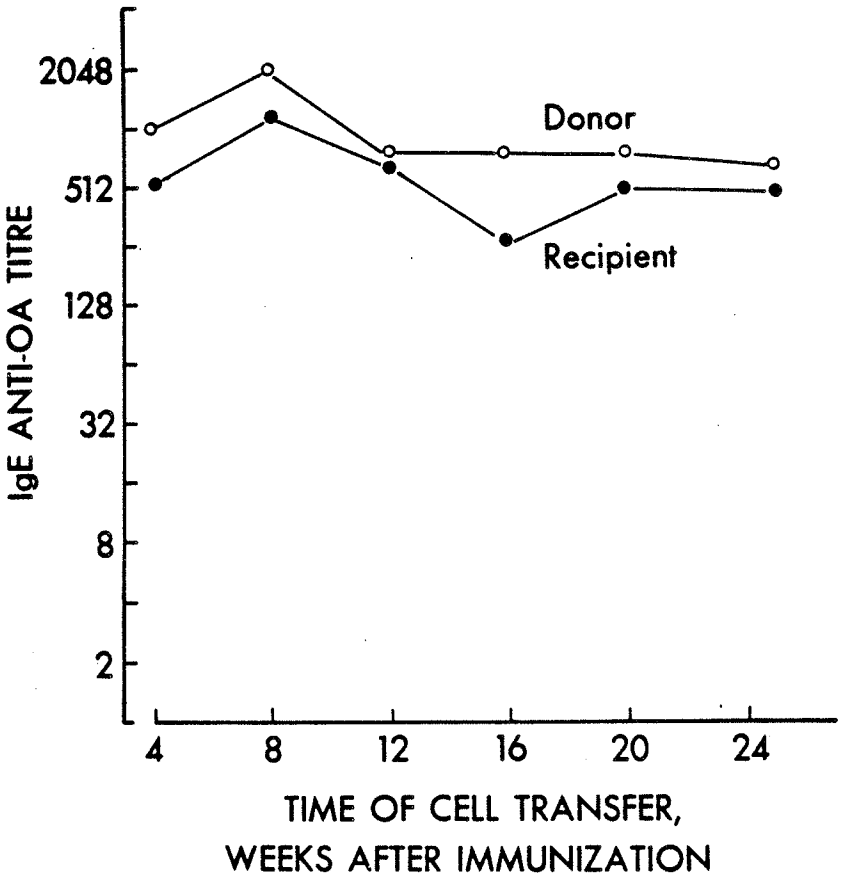


Figure 13.

The ability of primed spleen cells to transfer the adoptive anti-OA response up to eight months after immunization of donor mice. Seventy mice were immunized i.p. with 1 μg OA in 1 mg $Al(OH)_3$ . At monthly intervals after immunization, the spleens of six mice were used as a source of OA primed cells. Five x $10^7$ primed cells in Medium 199-20% FCS (Gibco C631107) were injected into each of a group of six irradiated recipients. The PCA (●) titres of the sera of recipient animals were determined 21 days after cell transfer. Also shown are the IgE anti-OA titres of donor sera at the time of cell transfer (o).

maximum IgE anti-OA titres generated in recipients of primed spleen cells were compared to the anti-OA titres of donors, it was evident that a good correlation existed between the IgE antibody titres of donors and recipients ($r=0.83$) (Figure 14).

No other correlation could be established between antibody titres in donors and those in recipients. There was no correlation between the hemagglutinating antibody titres in donors and recipients ($r<0.1$), nor was there a correlation between donor hemagglutinating antibody titres and the IgE anti-OA titres of recipients ($r<0.1$). Moreover, within the donor groups there was no correlation between the IgE titres and hemagglutinating antibody titres stimulated by immunization. However within the groups of irradiated recipient mice there was a good correlation ($r=0.82$) between adoptive IgE anti-OA and adoptive hemagglutinating anti-OA titres.

It had been demonstrated that a period of approximately 4 weeks after immunization was required before cells capable of supporting an adoptive anti-OA response started to appear in the spleen. Since at 2 weeks after immunization the IgE anti-OA titres in immunized mice were already at their highest level, it was considered possible that the cells which supported antibody production in the donor mice might reside in a site other than the spleen. The ability of cells from the mesenteric lymph nodes, which drained the site of immunization of donor mice, to support an adoptive IgE anti-OA response was therefore examined.

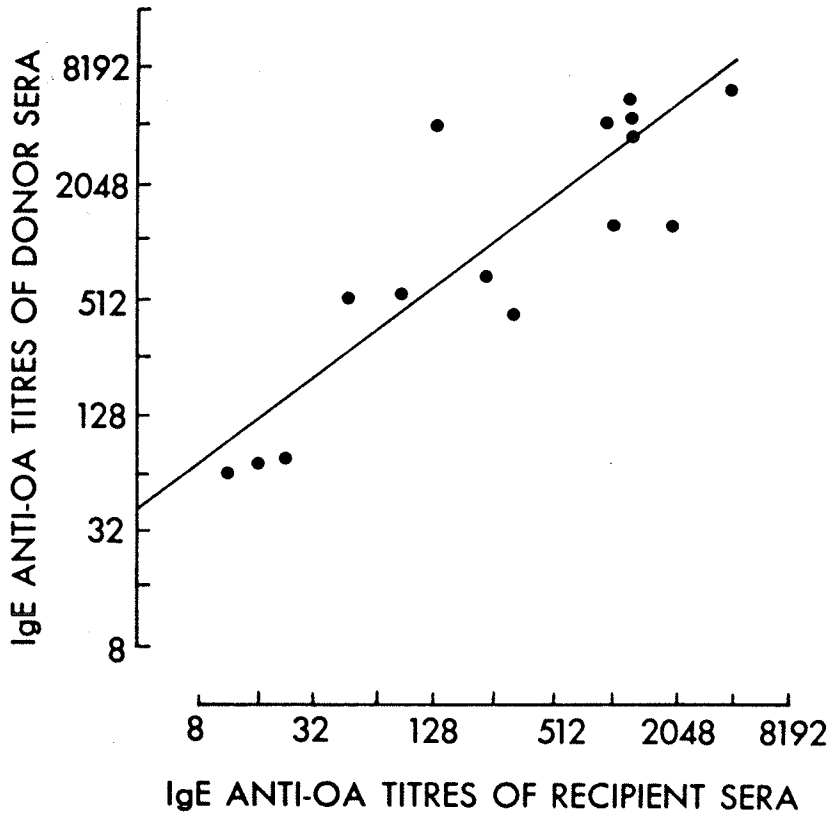


Figure 14.

Correlation of IgE anti-OA titres in the sera of spleen cell donors with the PCA titre in the sera of irradiated recipients. Spleen cells were transferred four to eight weeks after immunization of donors with 1 μg OA in $Al(OH)_3$ i.p. IgE anti-OA titres in the sera of donors were determined at the time of sacrifice and, in the sera of recipients, 21 days after cell transfer. ($r=0.83$, $p<0.001$)

At different times after immunization, mesenteric lymph nodes and spleens were removed from primed mice, suspended in Medium 199-20% FCS and injected, separately, into irradiated recipients. As had been seen with spleen cells (Table 2), lymphocytes from the mesenteric nodes were unable to support an adoptive IgE anti-OA response until 2 - 3 weeks after immunization of the donors. One month after immunization, however, cells from mesenteric nodes supported an adoptive IgE anti-OA response of the same magnitude as that generated by an equal number of primed spleen cells (Figure 15). Since the magnitude of the responses supported by both types of cells were comparable it would appear that the frequencies of cells capable of giving rise to adoptive anti-OA production were similar in both spleen and mesenteric lymph nodes.

### 3.4.4 Modulation of the transferred response by the recipient

Although the magnitude of the adoptive IgE anti-OA response was clearly dependent on a number of factors (number of cells transferred, time after immunization, donor antibody titre) contributed by the donor, the status of the irradiated recipients also contributed to the final anti-OA response. This first became evident when transfer of primed spleen cells to normal unirradiated mice did not lead to the production of anti-OA antibody in the recipient. Transfer of primed cells to normal mice was initially undertaken to establish whether

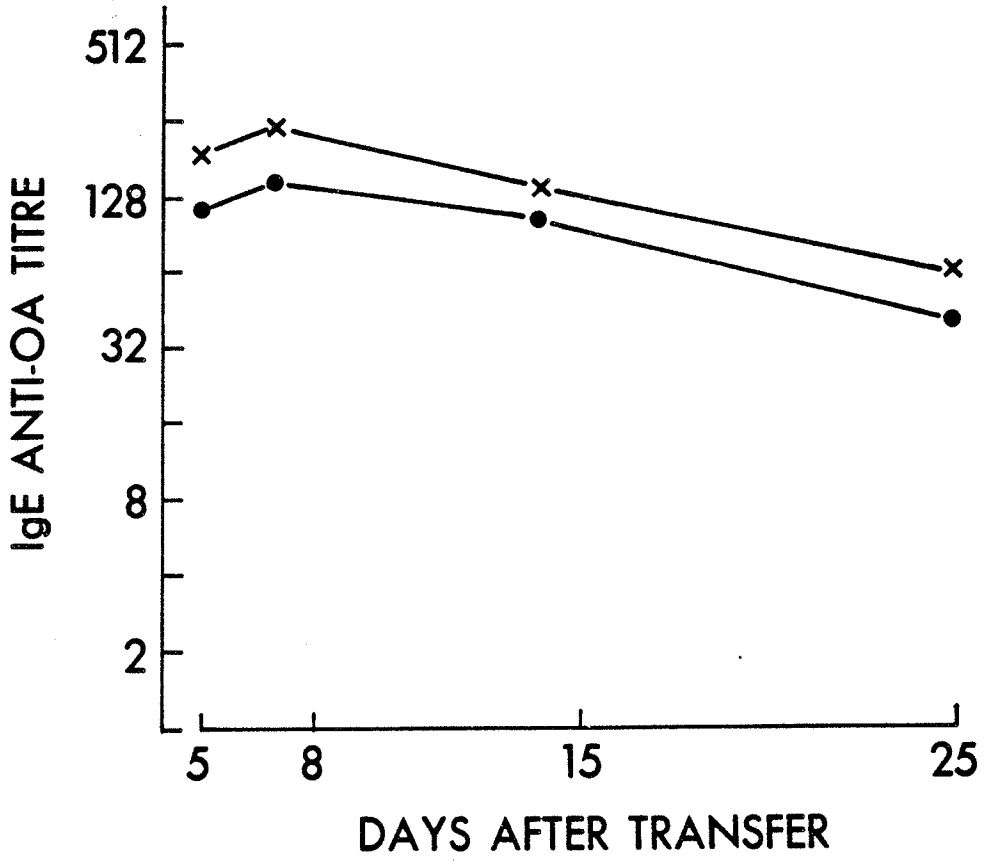


Figure 15.

The ability of lymph node cells to transfer the adoptive IgE anti-OA response to irradiated recipients. Spleen cells (•) or lymph node cells (x) were obtained from mice 28 days after immunization with 1 μg OA in $Al(OH)_3$ i.p. Cells were suspended in Medium 199 containing 20% FCS (Gibco C631107) and injected into irradiated mice at a dose of $5 \times 10^7$ cells/recipient.

the antigen independent transfer of an antibody response was similar to the more conventional adoptive transfer systems which require that the recipient be irradiated and challenged with antigen.

Since an intact recipient seemed to have the capacity to suppress the ability of primed spleen cells to produce an adoptively transferred response it was decided to investigate the suppressive function of the unirradiated animal.

Primed spleen cells were, therfore, transferred to recipients which had been exposed, 24 hours earlier, to doses of irradiation ranging from 0 to 900 Rads. The results are shown in Figure 16. Without irradiation of the recipient, no adoptive IgE anti-OA response was seen. Exposure of recipients to 150 Rads was sufficient to permit generation of an adoptive response. An increase in the magnitude of the response was facilitated by increasing the dose of irradiation upto 600 Rads. Above this dose no further increase was seen.

The ability of irradiated recipients to regenerate this suppressive capacity was demonstrated by transferring primed spleen cells into groups of recipients which had been exposed to 600 Rads at different times during the 9 days preceding adoptive transfer. Figure 17 shows that the adoptively transferred response is greatest if cells are injected into recipients within 12 hours of irradiation. With increasing time between irradiation and transfer, there is a gradual lessening of the adoptively transferred IgE

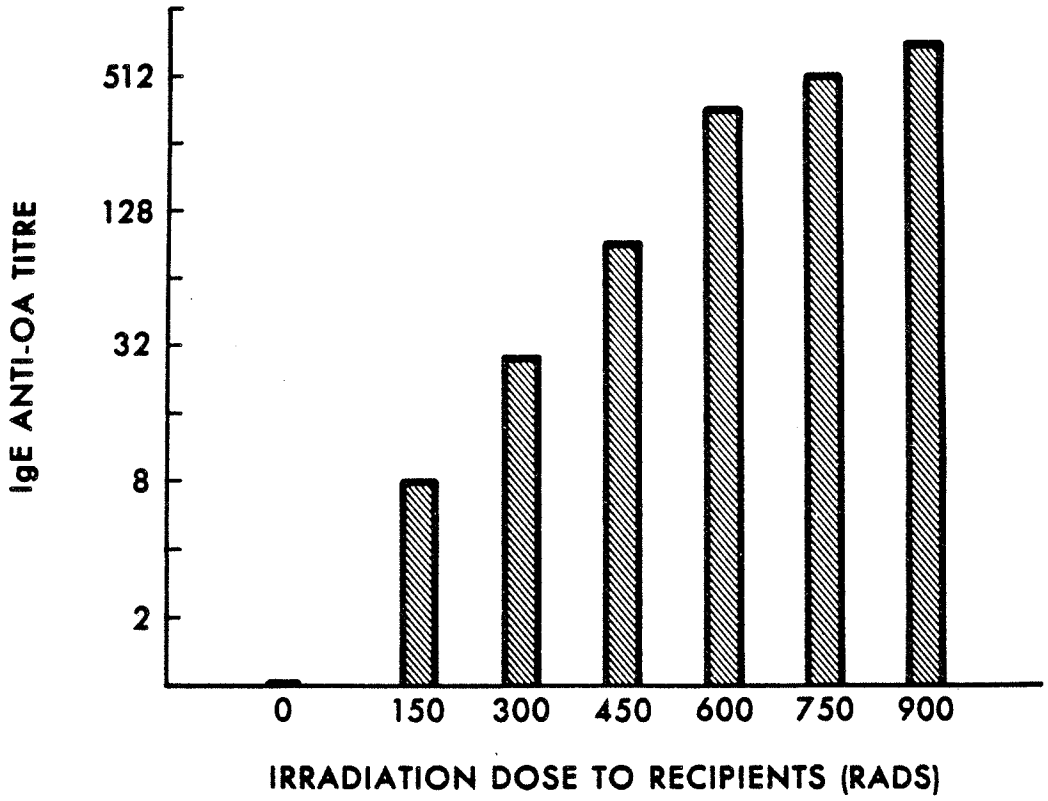


Figure 16.

The effect of irradiation dose, delivered to recipients, on the magnitude of the adoptive IgE anti-OA response. Groups of prospective recipients were exposed to increasing amounts of irradiation 20 hours before each mouse was injected with $5 \times 10^7$ OA-primed spleen cells in Medium 199-20% FCS. Results show the IgE anti-OA titres in the sera of recipients 19 days after transfer.

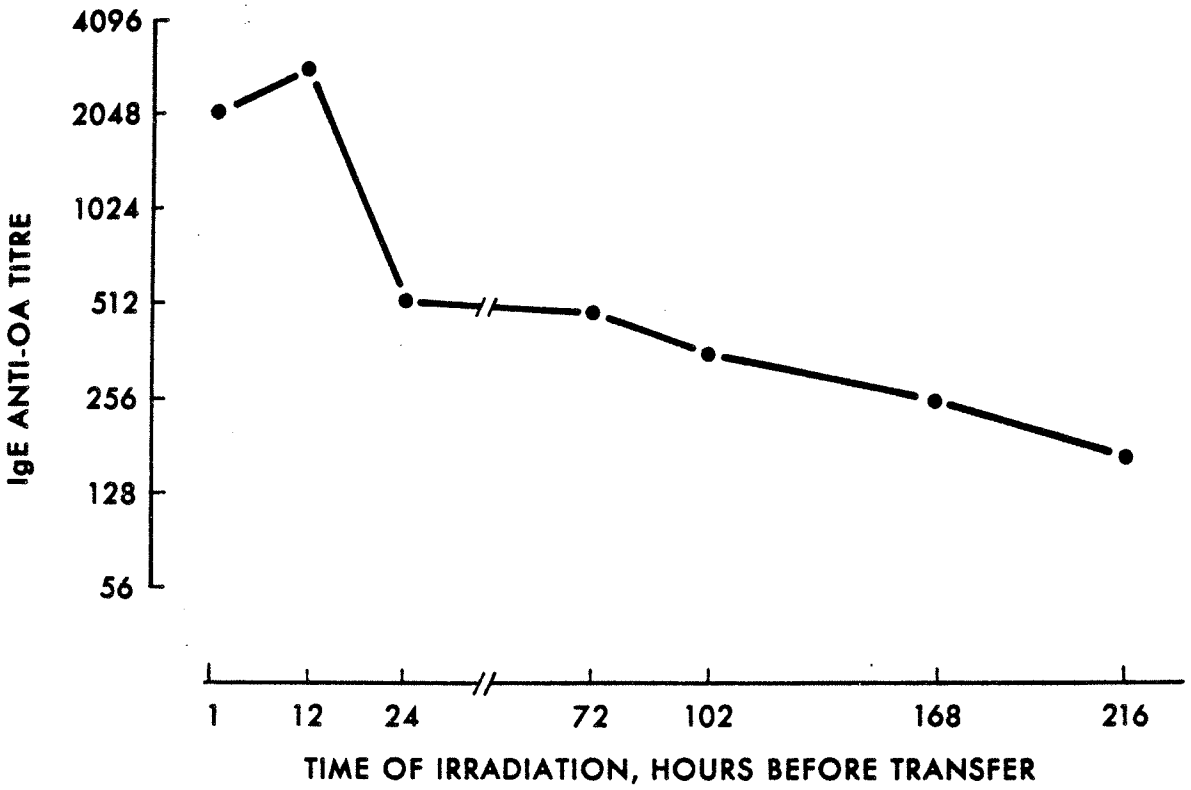


Figure 17.

Regeneration of the suppressive capacity of recipients with increasing time after irradiation. Separate groups of mice were irradiated with 600 Rads at one to 216 hr. (9 days) before each recipient received 5 x $10^7$ OA-primed cells from a single suspension of donor cells. Results show the IgE anti-OA titres in the sera of recipients 12 days after cell transfer.

anti-OA response. Experiments were carried out to determine if the inability of normal mice to support the adoptive response and the apparent regeneration of a suppressive effect with time after irradiation could be attributed to cells of the lymphoid system of normal animals. Chiorazzi et al had demonstrated the existence, in unimmunized mice, of a population of isotypes specific T cells that acted to suppress IgE antibody responses on subsequent immunization (104). To this end primed spleen cells were mixed with increasing numbers of spleen cells or thymocytes from normal mice and injected into irradiated recipients. The data are recorded in Table 3 and show that neither normal spleen cells nor thymocytes were able to suppress the adoptively transferred IgE anti-OA response.

## 3.5 Role of FCS in the adoptive anti-OA response

Since very few reports in the literature document the production of a secondary antibody response in the absence of an overt antigenic stimulus (133-136) it was important to examine the cell transfer system for any factor capable of stimulating antbody production. Fetal calf serum, which was routinely incorporated into the tissue culture medium used to isolate spleen cells for adoptive transfer, was examined because of its recognized role in supporting cell function particularly under in vitro conditions. Primed spleen cells were suspended in Medium 199 alone or in Medium 199 supplemented with normal mouse serum (NMS), bovine serum

TABLE 3

EFFECT OF NORMAL CELLS ON THE ADOPTIVE ANTI-OA RESPONSE

| Cells transferred | | IgE anti-OA titres in recipients (c*) |
|---|---|---|
| Primed Spleen (a*) | Normal cells (b*) | |
| $4 \times 10^7$ | 0 | 720 |
| " | $1 \times 10^7$ spleen | 610 |
| " | $2 \times 10^7$ " | 610 |
| " | $3 \times 10^7$ " | 640 |
| " | $4 \times 10^7$ " | 680 |
| " | $1 \times 10^7$ thymocytes | 540 |
| " | $4 \times 10^7$ " | 780 |

a*
Primed spleen cells were obtained from mice which had been immunized four weeks earlier with 1 µg OA in $Al(OH)_3$ i.p.

b*
Normal spleen cells and thymocytes were obtained from male BDF1 mice of the same age (14 weeks) as the donors of primed cells. Primed and normal cells were mixed in vitro in Medium 199 containing 20% FCS for injection into irradiated recipients.

c*
IgE anti-OA titres shown were determined 18 days after cell transfer.

albumin (BSA) or with one of two batches of FCS. Figure 18 indicates that in this experiment primed spleen cells prepared in medium alone did not adoptively transfer the IgE anti-OA response to irradiated recipients. A supplement of protein in the form of NMS or BSA was not sufficient to generate the adoptive response. FCS, Gibco batch number R461121, which had been used in most of the early experiments, supported the transferred IgE anti-OA response while Reheis FCS M41512, which had been identified for its low mitogenic effect on spleen cells in vitro, did not. As a result of these observations batches of FCS were designated as supportive or non-supportive of the adoptive IgE anti-OA response.

Figure 19 indicates that the magnitude of the adoptive IgE anti-OA response was dependent on the amount of FCS transferred with primed cells. Since mice received 5 x $10^7$ primed cells in a volume of 0.5 ml, a concentration of 20% FCS represents the injection of 100 ul of FCS per recipient.

Eleven different batches of FCS were tested for their ability to support the adoptive IgE anti-OA response. Table 4 indicates that a wide range of supportive capacities was found among the sera tested. Gibco sera R461121 and C631107 were identified as supportive sera. These two batches were used as such in experiments described in this thesis. Reheis batches M41512 and M50605 were recognized as non-supportive FCS. Batches of FCS that facilitated the IgE anti-OA response also supported the production of hemagglutinating antibody in irradiated recipients.

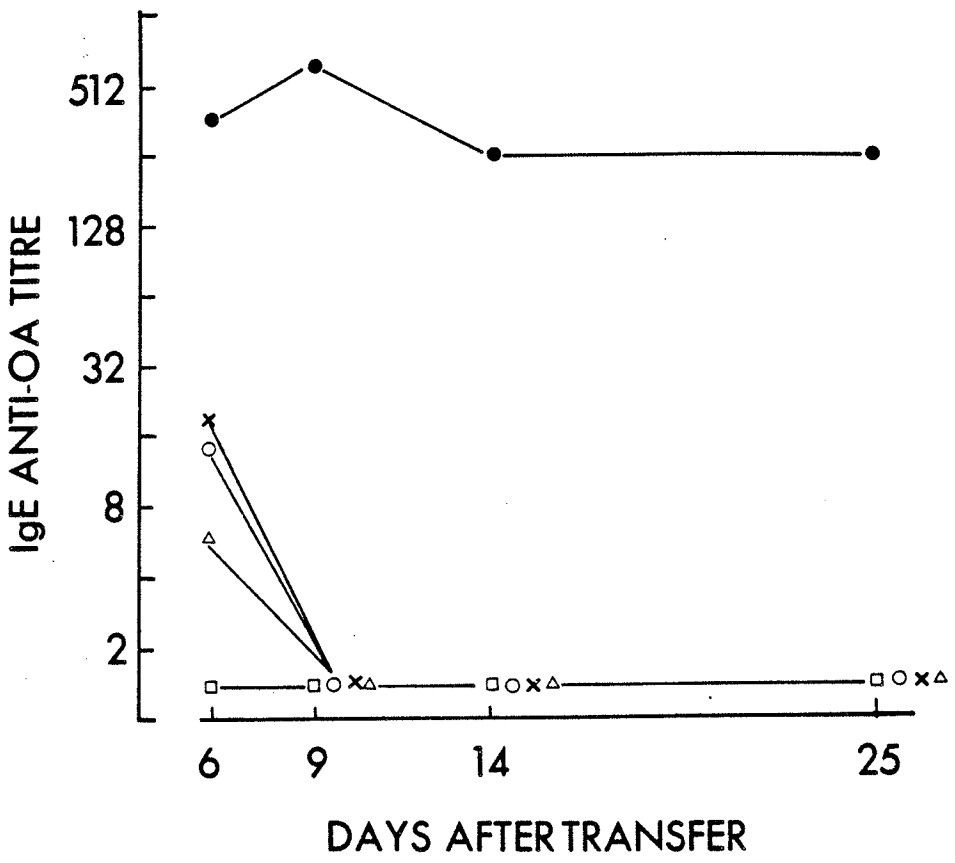


Figure 18.

The effect of FCS on the adoptive IgE anti-OA response. OA-primed spleen cells were prepared in Medium 199 alone (o) or, supplemented with 16% FCS, Gibco R461121 (•), with 16% FCS, Reheis M41512 (x), with 16% normal mouse serum (□) or with 4% (w/v) bovine serum albumin (Δ). Five x $10^7$ cells were injected into each of the irradiated recipients in the five experimental groups.

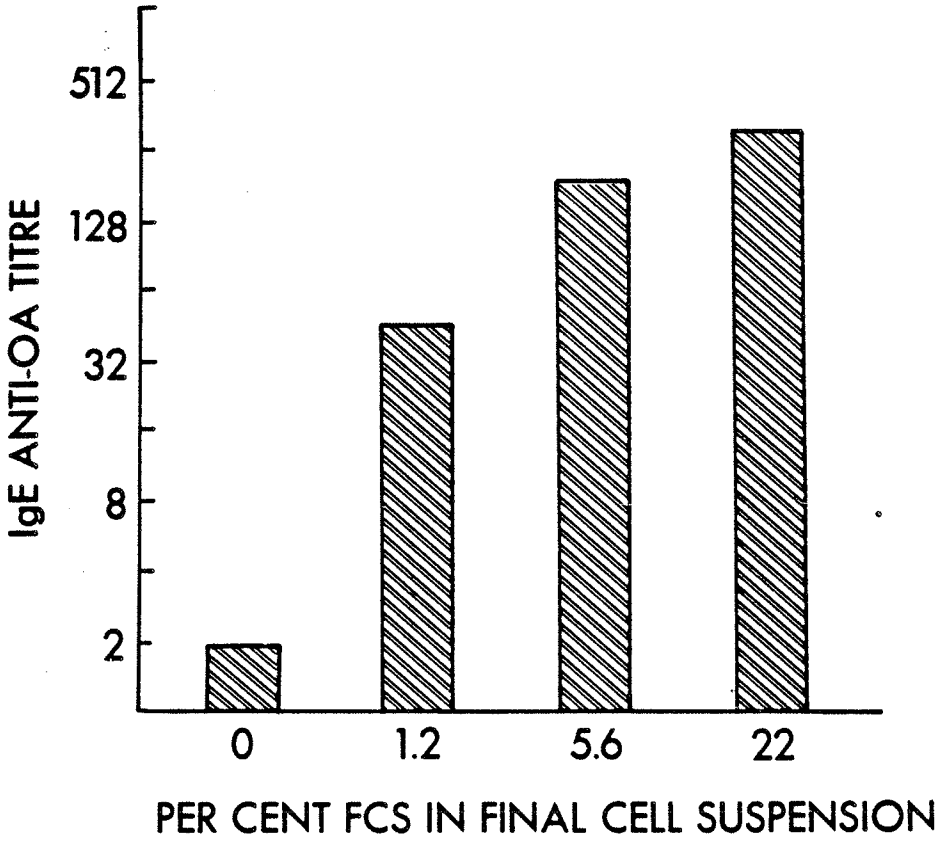


Figure 19.

The effect of increasing concentrations of FCS on the adoptive IgE anti-OA response. Irradiated recipients each received $5 \times 10^7$ OA-primed cells suspended in Medium 199 supplemented with increasing amounts of FCS, Gibco R46112. Results show IgE anti-OA titres in the sera of recipients nine days after cell transfer.

TABLE 4

SUPPORTIVE CAPACITY OF DIFFERENT BATCHES OF FCS

| Lot number | anti-OA Titre, Day 12 after transfer | |
|---|---|---|
| | PCA | HA |
| Gibco R461121 | 512 | 256 |
| R874221 | 32 | 32 |
| C173028 | 64 | 32 |
| C775120 | 128 | 32 |
| E076019 | 16 | 8 |
| A976616 | 0 | 2 |
| C631107 | 1024 | 128 |
| Reheis M41512 | 0 | 2 |
| M50605 | 0 | 2 |

Spleen cells were obtained from mice which had been immunized 4 - 8 weeks earlier with 1 $\mu$g $Al(OH)_3$ i.p. Irradiated recipients were injected i.v. with $5^3 \times 10^7$ cells suspended in Medium 199 containing 20% FCS. Anti-OA titres in the sera of recipients were determined by PCA and HA.

The role of FCS can be described as supportive rather than obligatory because, in a significant number of experiments to be described in section 3.5.4, no supplement of FCS was required to elicit an adoptive IgE anti-OA response in the absence of added OA.

Experiments were carried out to determine whether the stimulus provided by FCS could be effectively delivered to primed cells in vitro before adoptive transfer to irradiated recipients. Primed spleen cells were suspended either in Medium 199 alone or in medium supplemented with 20% supportive FCS. Duplicate aliquots of these suspensions were held either in a 37°C water bath or in ice water for 60 minutes. After incubation, half of the suspensions were washed and resuspended in Medium 199 before injection into irradiated recipients. Duplicate sets of suspensions were not washed but were injected directly into recipients. Table 5 shows that FCS was capable of supporting the adoptive IgE anti-OA response after only a brief exposure to primed cells in vitro. In vitro exposure of cells to FCS was found to be equally effective at 0°C and 37°C.

Three types of stimuli in FCS were considered to be possible sources of the supportive capacity of this reagent in cell transfer experiments. The first was the presence in FCS of a protein which cross reacted with OA. Such a protein would provide an antigenic stimulus to primed cells eliciting a secondary immune response in irradiated recipients. The second possible type of stimulus was a mitogenic one.

TABLE 5

in vitro EXPOSURE OF PRIMED CELLS TO FCS

| Spleen cell suspension | FCS | Incubation 60 min. | Wash Medium 199 | IgE anti-OA titre, Day 21 after transfer |
|---|---|---|---|---|
| 1 | - | 0°C | - | 0 |
| 2 | + | | - | 128 |
| 3 | - | | + | 0 |
| 4 | + | | + | 110 |
| 5 | - | 37°C | - | 0 |
| 6 | + | | - | 128 |
| 7 | - | | + | 0 |
| 8 | + | | + | 110 |

Primed spleen cells were obtained from mice which had been immunized 6 weeks earlier with 1 µg OA in 1 mg $Al(OH)_3$ i.p. Cell suspensions were made to a concentration of $5 \times 10^7$ /ml in Medium 199 and were supplemented with 20% supportive FCS, Gibco R61121, as indicated (+ or -). Cells were held either at 0°C or at 37°C for 60 minutes before the appropriate suspensions were washed (+) and resuspended in Medium 199. Each of 4 irradiated mice comprising a recipient group were injected with $3.5 \times 10^7$ cells. IgE anti-OA titres in the sera of recipients were determined by PCA.

Mitogen could act by inducing the polyclonal multiplication and/or differentiation of lymphocytes including antigen-primed cells, thereby increasing the number of antibody producing cells in the system to a level where their product would be easily detectable. The third possible type of stimulus in FCS could be an undefined supportive or growth factor, as have been described for a number of immune systems examined in vitro. Experiments were carried out to determine whether OA-like antigen or mitogenic factors could account for the ability of FCS to facilitate the production of IgE anti-OA by OA-primed cells on transfer to irradiated recipients.

### 3.5.1 FCS as a possible source of antigen

It would be expected that stimulation of primed cells by a cross reacting antigen at the time of adoptive transfer would lead to the production of a significant amount of antibody to that cross reacting antigen in the recipient animal. Experiments were designed to test whether the antibodies produced in irradiated recipients as a result of adoptive transfer of OA-primed cells in medium supplemented with FCS contained antibody to FCS.

Two groups of rats were each sensitized with anti-OA from two sources; from mice that had been immunized with 1 $\mu$g of OA in $Al(OH)_3$ and from irradiated mice that had been injected with $5 \times 10^7$ OA-primed cells in medium containing 20% FCS. One group of rats was challenged i.v. with OA and the second with FCS. The results are shown in Table 6. The

rats challenged with OA yielded PCA titres ranging from 420 to 4096 for the two different sources of sensitizing antisera, whereas the rats challenged with FCS failed to show any PCA activity.

The lack of antigenic similarity between OA and FCS evident from the inability of these two reagents to react with the same antibody was confirmed by in vitro neutralization experiments. Aliquots of mouse anti-OA serum were mixed with increasing amounts of OA, FCS, BSA or NMS and incubated at 37° C for 1 hour, followed by 18 hours at 4°C. Residual anti-OA activity in each aliquot was detected by PCA in rats and by hemagglutination of OA-coated SRBC. The data in Table 7 show that, as expected, OA inhibited completely both PCA activity and antibody mediated hemagglutination. Neither the PCA activity nor the hemagglutination titre of anti-OA sera was reduced by FCS. BSA at concentrations ten times greater than those used for OA had no effect on either PCA nor HA titres.

Inhibition of agglutination of OA coupled SRBC was first detectable at concentrations of 4 to 8 μg OA/ml. An equivalent concentration of cross reacting antigen would, therefore, have to be present in FCS to effect neutralization of antibody. As indicated earlier, irradiated recipients received primed spleen cells supplemented with 100 μl of FCS. The volume of FCS could, therefore, at most, contain 0.4 to 0.8 μg of cross reacting antigen and still escape detection in the neutralization assay. It became necessary to establish whether OA-primed cells, on adoptive transfer, were sensitive

TABLE 6

LACK OF ANTIGENIC SIMILARITY BETWEEN OA AND FCS IN PCA

| Source of sensitizing Mouse serum (a*) | PCA titre on challenge with: OA (b*) | FCS |
|---|---|---|
| Day 10 after immunization | 4096 | <2 |
| Day 21 after immunization | 1024 | <2 |
| Day 14 after cell transfer | 420 | <2 |
| Day 30 after cell transfer | 856 | <2 |

a*
The skins of rats were sensitized with pooled sera from mice immunized with 1 μg OA in $Al(OH)_3$ i.p. or from recipients of primed spleen cells suspended in medium containing FCS, Gibco R461121.

b*
Duplicate pairs of rats were challenged either with 1 mg OA or with 1 ml FCS, Gibco R461121, in 0.5% Evan's blue dye.

TABLE 7

NEUTRALIZATION OF ANTI-OA ACTIVITY

| | | Residual Anti-OA titres | | | | Residual Anti-OA titres | |
|---|---|---|---|---|---|---|---|
| | | PCA | HA | | | PCA | HA |
| OA | 2 µg/ml | 2048 | 64 | PBS | | 2048 | 80 |
| | 4 " | 1800 | 50 | | | | |
| | 8 " | 2048 | 6 | BSA | 312 µg/ml | 1600 | 80 |
| | 16 " | 2090 | 2 | | 625 " | 1400 | 80 |
| | 32 " | 400 | 0 | | 1250 " | 2048 | 80 |
| | 64 " | 64 | 0 | | 2500 " | 1020 | 80 |
| | 128 " | 45 | 0 | | 5000 " | 2048 | 80 |
| | 250 " | 32 | 0 | | 10000 " | 1400 | 80 |
| | 500 " | 8 | 0 | | 20000 " | 1800 | 80 |
| | 1000 " | 2 | 0 | | | | |
| FCS | 1/16 | 1800 | 80 | NMS | 1/16 | 1800 | 80 |
| | 1/8 | 1800 | 80 | | 1/8 | 1800 | 80 |
| | 1/4 | 2000 | 80 | | 1/4 | 1600 | 80 |
| | 1/2 | 1800 | 64 | | 1/2 | 1800 | 80 |
| | Undiluted | 1800 | 64 | | Undiluted | 1800 | 80 |

Pooled sera were obtained from irradiated recipients of primed spleen cells. Aliquots of 0.2 ml anti-OA serum were mixed with increasing quantities of OA, FCS, BSA or NMS. These were incubated at 37°C for 1 hour and at 4°C for 18 hours. Residual anti-OA activity was detected by diluting each sample in phosphate buffered saline and testing in PCA and HA assays.

to such low amounts of soluble antigen.

Primed spleen cells taken from mice 12 weeks after immunization were prepared in Medium 199 alone. Aliquots of the suspension were supplemented with OA at concentrations which resulted in the injection of 0.1 ng to 10 µg of antigen into groups of irradiated recipients. The results of this experiment are shown in Figure 20. The primed spleen cells were sensitive to unexpectedly small amounts of antigen. As little as 1.0 ng of OA stimulated the production of anti-OA in the irradiated recipient. Ten nanograms of OA stimulated a response equivalent to that seen with 20% FCS, indicating that cross reacting antigen at a concentration of 100 ng/ml would be sufficient to support the ransferred response. Since this concentration could not be detected by the neutralization procedure described earlier, experiments were carried out to remove any material that might be antigenically similar to OA.

Three 10 ml affinity chromatography columns were prepared as described in section 2.6.2. The first consisted of Sepharose-4B beads to which had been coupled rabbit anti-OA. The second column was Sepharose 4B to which was coupled rabbit antibody to goat immunoglobulin and the third column had been activated by cyanogen bromide and quenched by ethanolamine. To each column was applied 3.0 ml of supportive FCS, Gibco C631107, which was allowed to react with the columns for 90 minutes at room temperature. Materials which had not bound to the column were then washed off with PBS until the absorbance at 280 nm was zero. These

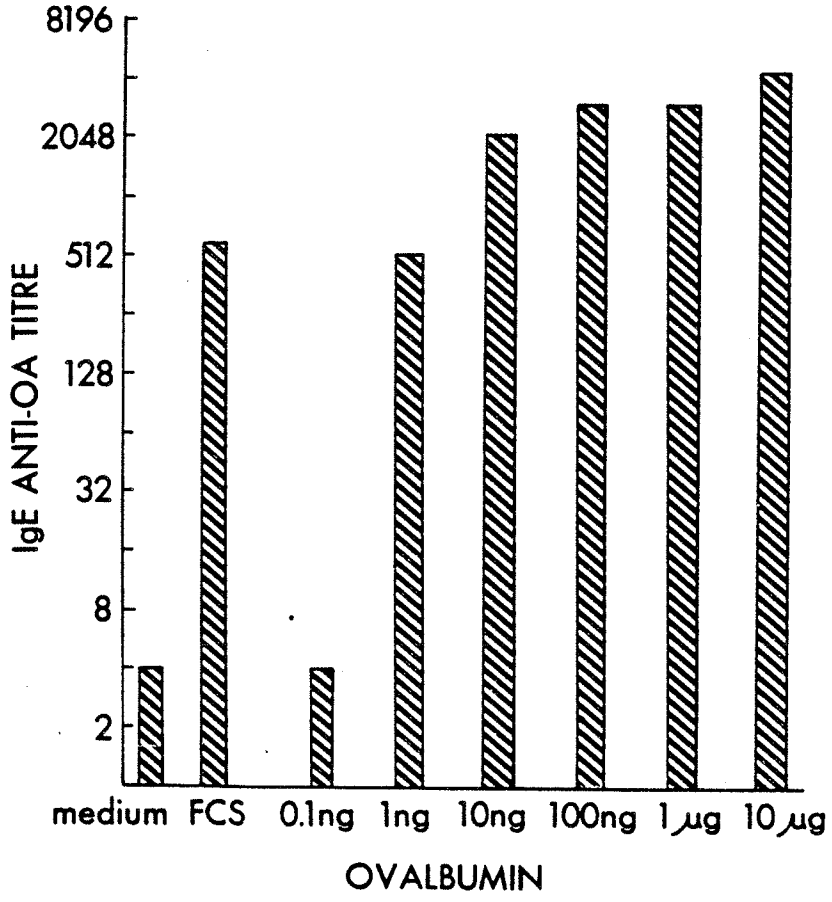


Figure 20.

Sensitivity of OA-primed cells to antigen. Primed spleen cells were taken from mice 12 weeks after immunization. Aliquots of the suspension were supplemented with 20% FCS, Gibco C631307, or with soluble ovalbumin at concentrations ranging from 0.1 ng to 10 µg per $5 \times 10^7$ cells before injection of this cell number into irradiated recipients. The IgE anti-OA titres shown were detected in sera obtained 10 days after cell transfer.

non-absorbed fractions were concentrated using Amicon PM10 membranes to the initial volume of 3.0 ml. Material which had bound to the columns was eluted with 0.2 M Glycine-HCl buffer at pH 2.2. Since the absorbance at 280 nm for these fractions was very low, 40 ml were collected from each column, neutralized with 0.1 N NaOH and concentrated to 3.0 ml using PM10 membranes. The six samples resulting from this procedure were used to supplement suspensions of OA-primed cells to a concentration of 20% (v/v) before transfer into irradiated recipients. Results are shown in Table 8 and, unfortunately, are rather equivocal.

The supportive capacity of non-adsorbed material from the anti-OA column was lower than that from the two control columns. At first sight this would indicate the absorption to the anti-OA column of an OA-like molecule. However, on elution of absorbed material with acid buffer all three columns yielded material of comparable supportive capacity. If OA-like material had been specifically absorbed to the anti-OA column only, one would have expected the other two columns to yield negligible supportive activity in the fractions eluted with acid.

Since we were unable to define conditions that would consistently lead to the production of high titres of anti-OA in irradiated recipients in the absence of FCS, it was decided to pursue further investigations using spleen cells suspended in supportive FCS. However, all experiments included a control group of recipient mice which were injected with

TABLE 8

BINDING OF THE SUPPORTIVE COMPONENT OF FCS
TO AFFINITY COLUMNS (a*)

| Column Type | $Log_2$ IgE anti-OA titres (c*) Non-Adsorbed | Adsorbed |
|---|---|---|
| Seph-4B-anti-OA | 4.5 | 7.0 |
| Seph-4B-anti-Ig | 7.8 | 9.4 |
| Seph-4B | 9.4 | 7.6 |
| Control Supplements (b*) | | |
| Medium 199 | 3.0 | |
| 20% FCS | 9.5 | |

a*
Three ml aliquots of supportive FCS, Gibco C631107, were applied to each of 3 affinity columns and fractionation was carried out as described in the Materials and Methods. Both the material that was not adsorbed to the column and the material eluted with 0.2 M Gly-HCl pH 2.2 (adsorbed) were concentrated to 3.0 ml. These fractions were used at final concentrations of 20% (v/v) to supplement suspensions of OA-primed cells for injection into irradiated recipients.

b*
Control cell suspensions were supplemented either with additional medium or with 20% FCS, C631107, for injection into irradiated recipients.

c*
Anti-OA titres were determined in samples taken 10 days after adoptive transfer.

primed spleen cells prepared in medium 199 alone. This ensured that the experimental results could be interpreted with a knowledge of the type of response, whether FCS independent or FCS dependent, that had been elicited. The following three sections of this thesis contain results, on the role of antibody and the cooperation of B and T cells in the adoptively transferred response, which were obtained from experiments controlled as just described. When a group of donor mice whose cells were capable of supporting a FCS independent adoptive response were identified, experiments were carried out to determine whether the humoral and cellular factors affecting the FCS independent responses were the same as those active in the adoptive response supported by FCS. Detailed evidence for the similarity of mechanisms underlying the adoptive anti-OA response in the presence and absence of FCS will be presented at the end of section 3.8.

### 3.5.2 FCS as a possible source of mitogen

Having reduced the likelihood that the supportive capacity of FCS was attributable to contamination with conventional antigen, evidence was sought for the presence of mitogenic activity in the serum.

To test directly for mitogenic activity in FCS, the ability of supportive FCS to stimulate incorporation of $^{3}$H-Thymidine into spleen cells was assessed. Spleen cells from normal BDF1 mice were cultured in microtitre plates in the presence of six different batches of FCS. The

incorporation of $^{3}$H-Thymidine was measured following cell harvest 60 hours after initiation of the cultures. The same six batches of FCS were used to supplement suspensions of OA primed cells before injection into irradiated recipients in order to compare their in vitro mitogenicity to their ability to support the adoptive anti-OA response. Results are shown in Table 9. Although the stimulation index for Gibco R461121 in culture was the highest and this serum also supported most effectively the transferred response, there was no overall correlation between the in vitro mitogenic capacity of the different sera and their ability to support an adoptive response. Final concentrations of 5% or 10% FCS in culture gave very similar stimulation indices.

These in vitro experiments implied that mitogens present in FCS did not play a significant role in the adoptive IgE anti-OA response. However, given the large number of factors that affect cellular responses in vitro (15), it was conceivable that the conditions used may have been inadequate to reveal the presence of such mitogens. Consequently, we chose to investigate directly the effect of several defined mitogens in the antigen independent adoptive transfer system. Primed spleen cells were suspended with increasing amounts of Lipopolysaccharide (LPS), dextran sulphate (DS), pokeweed mitogen (PWM), Concanavalin A and phytohemagglutinin (PHA). Each mitogen was initially tested over a 500-fold range of concentrations which included doses that had been shown to induce lymphocyte proliferation in vitro. In some cases, the

TABLE 9

in vitro MITOGENICITY OF FCS

| FCS, Lot No. | 3-H Thymidine Uptake (a*) cpm ± S.D. | Stimulation Index | Anti-OA PCA Titre, day 18 after transfer (b*) |
|---|---|---|---|
| None | 349 ± 83 | 1 | 0 |
| Gibco R461120 | 10720 ± 2061 | 30.1 | 256 |
| Reheis M50605 | 5508 ± 560 | 15.8 | 0 |
| Reheis M41512 | 5172 ± 846 | 14.8 | 0 |
| Gibco R874221 | 3708 ± 247 | 10.6 | 64 |
| Gibco C173028 | 2292 ± 474 | 6.6 | 32 |
| Gibco C77120 (c*) | 3427 ± 887 | 9.8 | 32 |

a*
Mitogenicity of each lot of FCS was tested by monitoring the uptake of 3-H Thymidine by cultures of normal BDF1 spleen cells as described in Materials and Methods. Results are expressed as mean cpm of quadruplicate cultures ± S.D. Results are those from cultures containing a final concentration of 5% FCS.

b*
Spleen cells from BDF1 mice immunized 6 weeks earlier with 1 ug OA with $Al(OH)_3$ i.p. were suspended in media supplemented with the different batches of FCS to a concentration of 17% and were injected into groups of irradiated recipients.

c*
FCS Lot No. C77120 was identified, by the manufacturer, as capable of supporting a primary in vitro anti-SRBC response in a Mishell-Dutton culture system.

upper limit of mitogen concentration was determined by the toxicity of the reagent either on the suspended spleen cells or in the irradiated recipient. Dextran sulphate at a concentration of 80 µg per $5 \times 10^7$ cells caused cell death resulting in a gelling of the cell suspension. LPS at a concentration of 200-400 ug/ recipient caused a transient weakening and weight loss in irradiated animals. This was first evident 4-5 days after transfer of cells suspended in LPS. Approximately one-fifth of the animals died as a result of the treatment. By day 10 after transfer the surviving mice appeared to have recovered fully.

The effects of suspending OA-primed cells in Medium 199 supplemented with increasing amounts of different mitogens are recorded in Figure 21. The top left hand panel shows again the direct relationship that existed between the amount of FCS added to the primed cells and the magnitude of the transferred response. On examining the response to specific mitogens, it was observed that LPS, DS and PWM were all capable of supporting a modest IgE anti-OA response in irradiated recipients. Neither of the T cell mitogens, Con A nor phytohemagglutinin, supported IgE anti-OA production. To determine whether, indeed, cell proliferation was a necessary step in the production of the adoptive IgE anti-OA response primed spleen cells were treated with the mitotic inhibitor, mitomycin C, before transfer into irradiated recipients. In vitro exposure to mitomycin C did not affect cell viability. As shown by Figure 22 exposure of cells to the mitotic

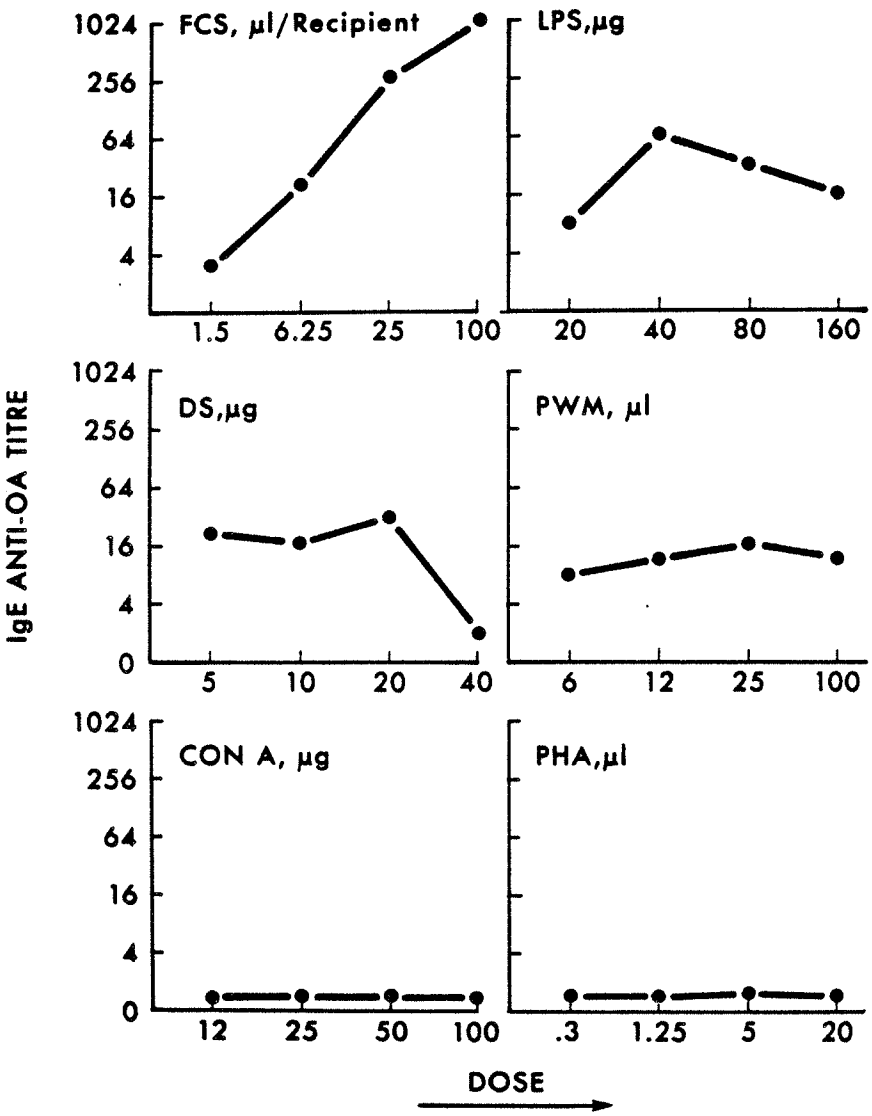


Figure 21.

The effects of the mitogens LPS, DS, PWM, Con A and PHA on the adoptive IgE anti-OA response. Aliquots of a single suspension of OA-primed spleen cells were supplemented with increasing amounts of FCS or of the mitogens, as indicated, before injection into irradiated recipients. The results shown indicate the IgE anti-OA titres in the sera of recipients 12 days after cell transfer.

inhibitor completely inhibited the production of both IgE and HA to OA in recipient mice indicating that cell replication was necessary for the establishment of adoptive antibody production.

3.5.3 Characterization of the supportive factor in FCS

Aliquots of a batch of FCS, Gibco C631107, which had been shown to support adoptive anti-OA production in the absence of antigenic challenge to recipients were tested as outlined in Table 10. The supportive activity was unchanged in FCS maintained at 4°C for 72 hours and was unaffected by heating the FCS to 56°C for 60 minutes. The repeated freezing, by immersion in an alcohol-dry ice bath, and thawing, at room temperature, of an aliquot of FCS over a period of 8 hours did not affect its ability to support the adoptively transferred anti-OA response. The supportive capacity also persisted in FCS that had been dialyzed against large volumes of PBS. This last observation indicated either that the supportive factor had a molecular weight greater than 10,000 or that it was associated with some serum protein of that size or larger.

In an attempt to determine the molecular weight of the supportive factor in FCS, an aliquot of FCS was fractionated on a column of Sephacryl A-5M which had been calibrated previously with blue dextran, ferritin, catalase, IgG, BSA and myoglobin. Figure 23 shows that such a column was capable of resolving solutes with relative molecular

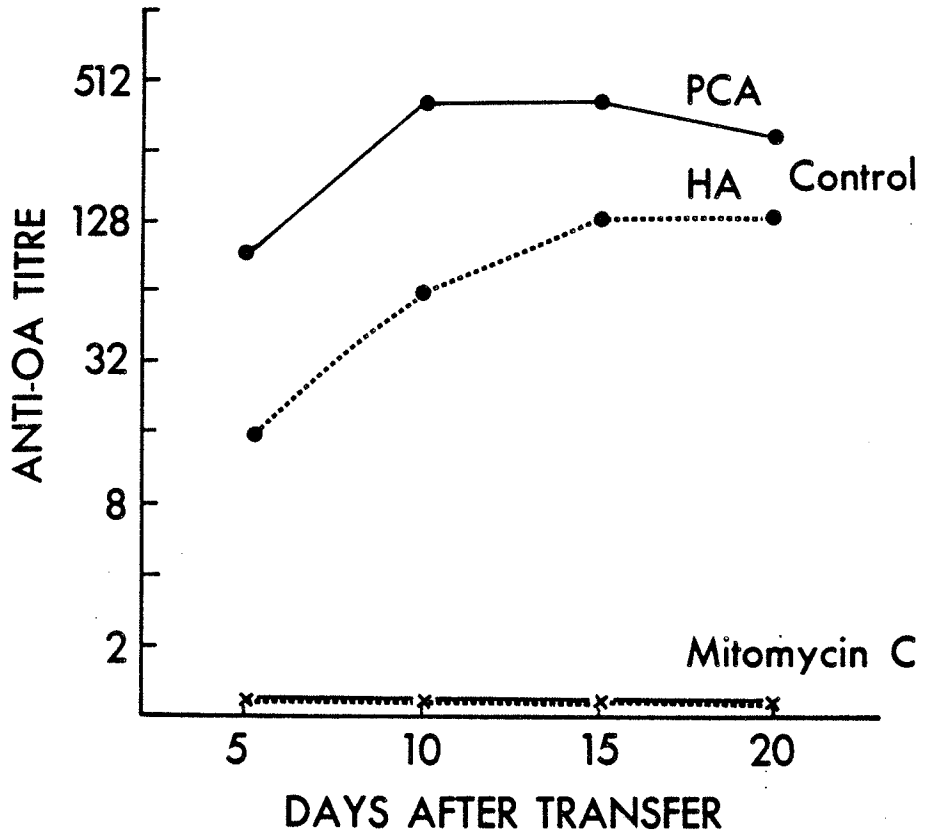


Figure 22.

The effect of exposure of primed cells to Mitomycin C on the adoptive anti-OA response. OA-primed spleen cells were suspended either in Medium 199 alone (•) or in medium supplemented with Mitomycin C (x) to a concentration of 40 μg/ml/5 x $10^7$ cells. Suspensions were incubated at 37° C for 30 min., washed and resuspended in Medium 199-20% supportive FCS for injection into irradiated recipients. The sera of recipients were assayed for both IgE antibody (——) and HA (---) to OA.

TABLE 10

STABILITY OF THE SUPPORTIVE CAPACITY OF FCS

| Treatment of FCS (a*) | Adoptive IgE anti-OA titre (b*) | |
|---|---|---|
| | Day 10 | Day 20 |
| No treatment | 300 | 512 |
| 56 °C x 60 min. | 512 | 512 |
| 4 °C x 72 hr. | 300 | 220 |
| Freeze/thawed x 10 | 300 | 512 |
| Dialyzed x 72 hr. | 220 | 220 |
| No FCS | 4 | 4 |

a*
Five ml aliquots of FCS, Gibco C631107, were treated as indicated. Dialysis was carried out against a total of 12 litres of PBS at 4 °C.

b*
Primed spleen cells were obtained from mice which had been immunized 4 weeks earlier with 1 µg OA in $Al(OH)_3$ i.p. Suspensions were prepared in Medium 199, supplemented with the different FCS to a final concentration of 20%, and injected i.v. into irradiated recipients.

Results show the IgE anti-OA titres in the sera of recipients, as determined by PCA, on days 10 and 20 after transfer of cells.

masses ranging from $2 \times 10^4$ to $2 \times 10^6$.

Five ml of supportive FCS were applied to the same chromatography column and eluted with PBS. Eluted material was collected in volumes of 5 ml/tube. The elution profile, as determined by absorbance at 280 nm is recorded in Figure 24. The contents of the first 20 eluate tubes were pooled and labelled fraction A. Each successive series of 20 tubes were also pooled to constitute fractions B, C, D, and E. A control sample, F, was prepared by diluting 5.0 ml of FCS to 100 ml. Fractions A, B, C, D and F were each concentrated to 4.0 ml using an Amicon PM10 membrane. Because of the smaller molecular weight of the contents of fraction E, this pool was concentrated using an Amicon UM-2 membrane.

Each of the concentrated fractions was used to supplement a suspension of OA-primed cells for injection into irradiated recipients. Results are shown in Table 11. Fraction A, which was roughly the void volume of the column and Fraction E, which contained material of the smallest molecular weight (upto 30,000) supported a very low adoptive response. Fractions B, C and D appeared equally potent in supporting the transferred anti-OA production. The adoptive responses supported by these fractions were, in all cases, a little lower than those produced by control samples.

### 3.5.4 FCS independent transfer of the anti-OA response

As has been indicated earlier, the role of FCS in the system under examination is considered supportive rather

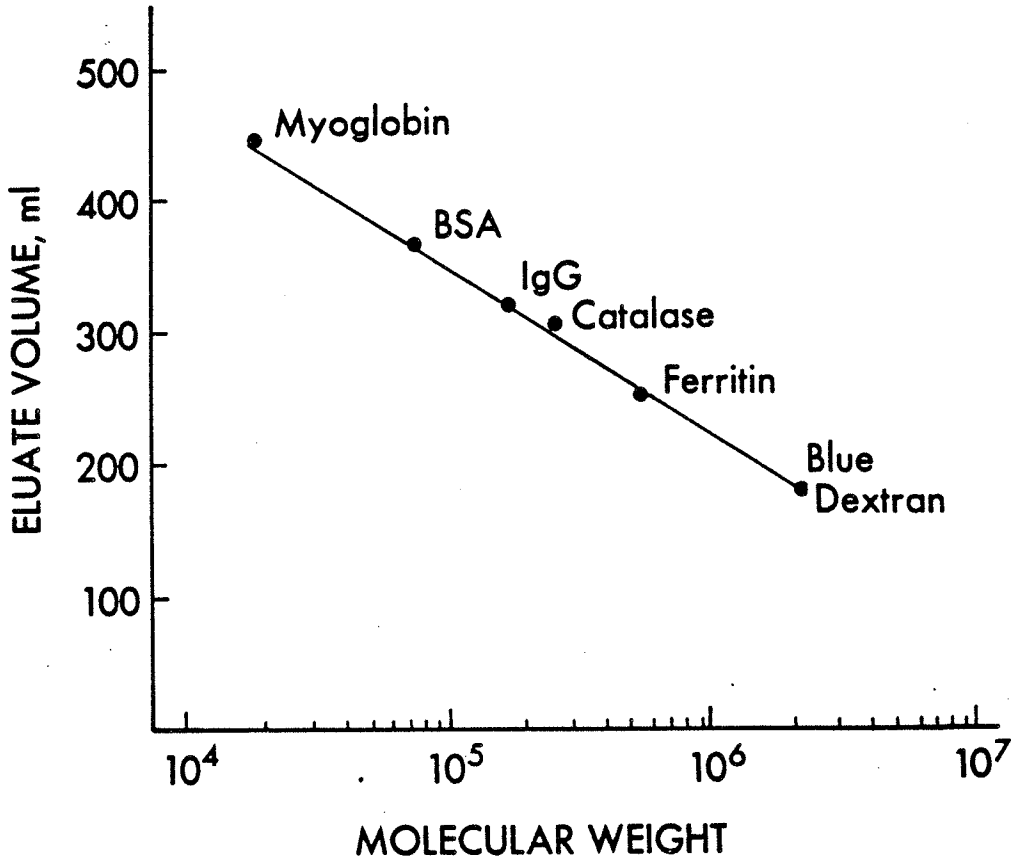


Figure 23.

Calibration of an A-5M Sephacryl chromatography column. A sample of 6 ml containing 20 mg ferritin, 20 mg catalase, 20 mg bovine gamma globulin, 20 mg bovine serum albumin, 20 mg whale skeletal muscle myoglobin and blue dextran was applied to a column measuring 2.6 x 90 cm. PBS was the eluting buffer. The flow rate was 20 ml/hr. Eluates of 5.0 ml were collected and their absorbance at 280 nm was determined. Results were expressed by plotting the elution volume of each marker against its known molecular weight.

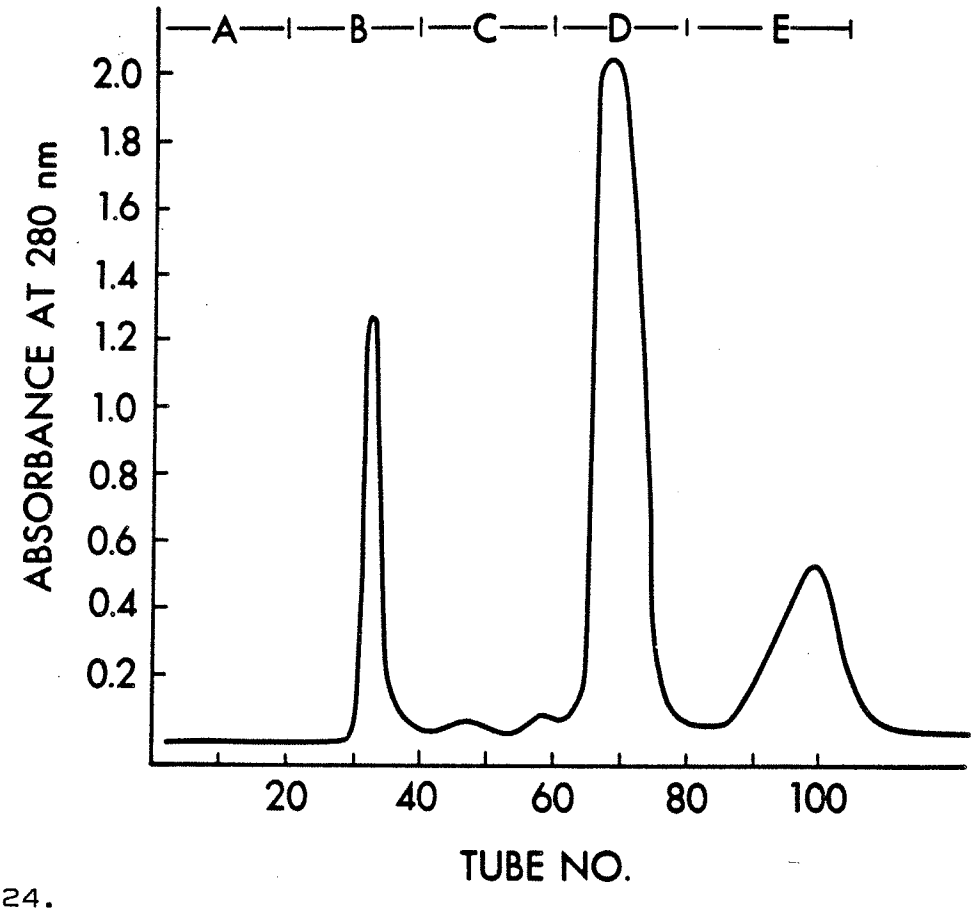


Figure 24.

Fractionation of FCS on a column of A-5M Sephacryl. Five ml of supportive FCS, Gibco C631107, were applied to a column (2.5 x 90 cm) and eluted with PBS at a rate of 20 ml/hr. Eluates were collected in 5.0 ml volumes. Each successive group of 20 eluates was pooled and designated fraction A, B, C, D or E. Fractions A, B, C and D were each concentrated to 4.0 ml using an Amicon PM-10 membrane. Fraction E was concentrated using a UM-2 membrane. A control fraction, F, was prepared by diluting 5.0 ml of FCS to 100 ml and concentrating it to 4.0 ml using a PM-10 membrane.

TABLE 11

SUPPORTIVE CAPACITY OF A-5M FRACTIONS OF FCS

| Fraction (a*) | Adoptive IgE anti-OA titre (b*) | |
|---|---|---|
| | Day 8 | Day 19 |
| A | 16 | 16 |
| B | 210 | 200 |
| C | 256 | 256 |
| D | 256 | 256 |
| E | 16 | 16 |
| F | 512 | 512 |
| Untreated FCS | 800 | 450 |
| Nil | 64 | 10 |

a*
Fractions A to F were obtained as described in the legend to Figure 23.

b*
Primed spleen cells were obtained from mice which had been immunized 5 weeks earlier with 1 μg OA in $Al(OH)_3$ i.p. Suspensions were prepared in Medium 199 supplemented with the appropriate fractions to a concentration of 20% (v/v), and injected i.v. into irradiated recipients. Results show the IgE anti-OA titres in the sera of recipients on days 8 and 19 after transfer of cells.

than obligatory since in a significant number of experiments, IgE anti-OA was detected in the sera of irradiated mice which had received cells suspended in Medium 199 alone. In about 30% of experiments, the FCS independent, adoptively transferred response was 25 to 50% of that generated on the addition of FCS to OA primed cells (Figure 25). In addition, it can be seen that the spontaneously generated anti-OA response generally paralleled the FCS dependent response and persisted for many weeks after transfer. In most of the remaining experiments, a low level of IgE anti-OA production (titres of 2 to 32) could usually be detected in mice which had received cells that had not been exposed to FCS or antigen.

Given the sensitivity of the OA primed cells to antigen, precautions were taken to minimize possible stimulation of cells by OA which might contaminate glassware and pipets. The use over a sustained period of new, sterile, disposable plastic tubes and pipets had no effect on the incidence of spontaneous production of IgE anti-OA in irradiated recipients.

The possibility that irradiated mice were exposed to OA or to cross reacting antigen in their food was considered. A saline extract prepared from the mouse chow Wayne Lab-Blox did not react in Ouchterlony double diffusion gels with anti-OA raised in hyperimmunized rabbits. Moreover, the manufacturer of the feed stated that no poultry products were incorporated into the pellets.

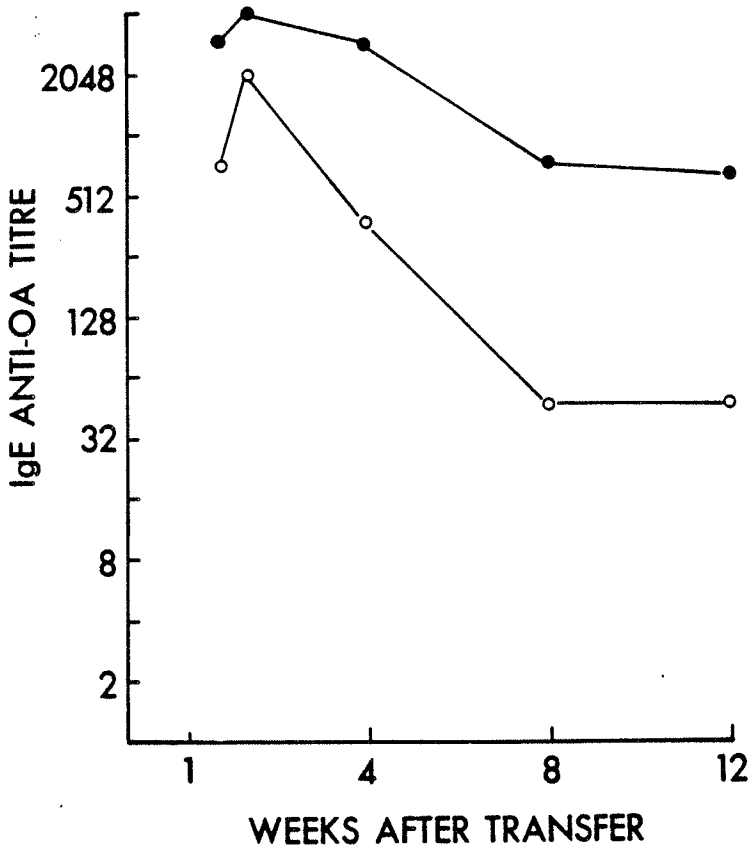


Figure 25.

FCS independent transfer of the IgE anti-OA response. IgE anti-OA responses generated by adoptively transferred OA-primed cells in the presence (●) and absence (o) of supportive FCS were monitored for 12 weeks.

Since the existence of a system in which antibody production by primed cells was independent of any exogenous stimulus was particularly attractive for the investigation of long term humoral responses, attempts were made to define conditions that would favour the consistent expression of a spontaneous response.

Mitchison and Kontiainen had stated that incubation of primed cells at 37°C was necessary to generate an antigen independent response to fowl gamma globulin in their experimental systems. It had already been demonstrated that incubation at 37°C was not required for the FCS dependent transfer of IgE anti-OA production (Figure 5, Table 5). Incubation of OA-primed cells at 37°C for up to three hours in Medium 199 alone did not consistently result in a significant spontaneous transfer of antibody production. Moreover, primed spleen cells that had been held at 0 to 4°C for no longer that 45 minutes from the time of sacrifice of the donors to the time of injection into irradiated recipients sometimes produced IgE anti-OA titres of 500 to 1000 in irradiated recipients.

Figure 17 had shown that the magnitude of the FCS dependent, adoptive IgE anti-OA response was proportional to the dosage of irradiation delivered to the recipients. To determine whether the dosage of 600 Rads chosen for most of the investigations was on the edge of some critical plateau for the FCS independent response, the effect of irradiation dose to the recipients on the magnitude of the spontaneously

transferred response was investigated. Figure 26 reveals that as with the FCS dependent response, the spontaneous IgE anti-OA titres depended on the dose of irradiation delivered to recipients. However, doses of 450 to 750 Rads allowed the production of equivalent titres of IgE anti-OA, indicating that 600 Rads was not a critical dose for the generation of a spontaneous response. In contrast to the FCS dependent response, the spontaneous production of IgE anti-OA did not occur in recipients that had been exposed to 150 or 300 Rads.

It has been suggested that aging is associated with a loss of suppressor cell function (137). The possibility that the spontaneously transferred IgE anti-OA response was due to such an age dependent suppressor cell defect was investigated. Primed spleen cells from a single group of donors were transferred into irradiated recipients of different ages. Table 12 indicates that at ages ranging from 8 to 26 weeks recipients supported both FCS dependent and spontaneous responses. There was no appreciable variation with age in the magnitude of both types of responses. Cells taken from donors from 3 to 8 months of age also did not show a consistent age related change in their ability to produce a FCS independent response.

One experiment did indicate that successful spontaneous transfer of high titred production of IgE anti-OA was associated with the donor population of cells. Mice from a group (A) of immunized animals whose spleen cells had earlier, unexpectedly, produced high titres of IgE anti-OA in

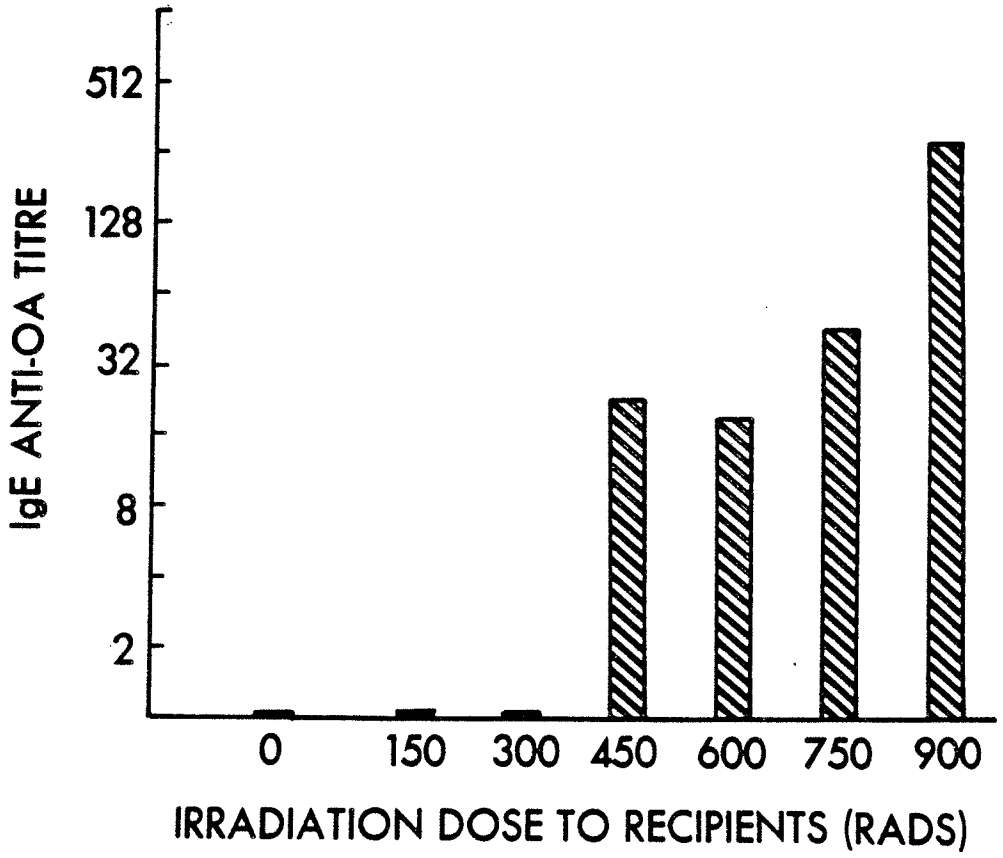


Figure 26.

The effect of irradiation dose delivered to recipient mice on the spontaneous transfer of IgE anti-OA production. Groups of prospective recipients were exposed to increasing amounts of irradiation 20 hr. before each mouse was injected with $5 \times 10^7$ OA-primed cells in Medium 199 alone. Results show IgE anti-OA titres in the sera of recipients 19 days after cell transfer. Figure 16 shows the FCS-dependent adoptive response generated by OA-primed cells from the same starting suspension.

TABLE 12

EFFECT OF AGE OF IRRADIATED RECIPIENTS ON THE ADOPTIVE IgE ANTI-OA RESPONSE

| Age of recipients (a*) weeks | $Log_2$ IgE anti-OA titre, Day 12 after transfer | |
|---|---|---|
| | FCS-Dependent | Spontaneous |
| 8 | 9.4 | 5.1 |
| 14 | 9.4 | 6.4 |
| 18 | 11.3 | 7.6 |
| 22 | 9.4 | 5.5 |
| 26 | 9.5 | 5.3 |
| Age of Donors (b*) | | |
| 12 | 9.0 | 1.0 |
| 16 | 10.5 | 4.0 |
| 20 | 9.5 | 2.0 |
| 24 | 8.0 | 2.0 |
| 28 | 10.0 | 4.0 |
| 32 | 9.5 | 4.5 |

a*
Prospective recipients of different ages were irradiated with 600 Rads and, 20 hours later, were injected with primed spleen cells obtained from a single group of immunized donors. Half the recipients in each age group received cells suspended in 20% supportive FCS (FCS-dependent response) and half received cells suspended in Medium 199 alone (spontaneous transfer).

b*
A large group of mice was immunized with 1 μg OA in $Al(OH)_3$ i.p. At monthly intervals after immunization, 5 - 6 mice were sacrificed and their spleens were used as sources of OA-primed cells. These cells suspended either in Medium 199 alone or in medium containing 20% FCS for injection into irradiated recipients.

the absence of FCS were used as donors of primed cells which were transferred into two groups of irradiated recipients differing only by four weeks of age. Spleen cells were also taken from a second group (B) of immunized mice for transfer into the same two recipient groups. Table 13 shows that donor group A clearly supported spontaneous IgE anti-OA production in both recipient groups, while cells from donor group B supported only FCS dependent responses.

## 3.6 Effect of antibody on the adoptive anti-OA response

The effect of anti-OA on the adoptive IgE anti-OA response was investigated for two reasons. First, since antibody would be expected to exert its specific effect by interacting with antigen, it was argued that any effect of antibody would indicate the presence of antigen in the system. It was necessary to determine whether antigen was present before postulating any role which it might have in the continued recruitment of cells to antibody production in the long term IgE anti-OA response.

Secondly, determination of whether antibody had an effect on the magnitude of the adoptive IgE anti-OA response would allow an examination of the possibility that hemagglutinating antibody to OA, which co-exists with IgE anti-OA in the circulation of immunized mice, might play a regulatory role during the ongoing IgE response. Experiments were carried out to determine whether the antibody present in donor serum could indeed affect the adoptive IgE anti-OA

TABLE 13

ASSOCIATION OF SPONTANEOUS TRANSFER WITH THE DONOR CELL POPULATION

| Donor Group | Recipient Group | Adoptive IgE anti-OA titre, Day 9 after transfer | |
|---|---|---|---|
| | | FCS-Dependent | Spontaneous |
| A (a*) | X | 4096 | 2048 |
| | Y | 4096 | 1024 |
| B | X | 2048 | 0 |
| | Y | 1024 | 0 |

a*
Mice from donor group A had, in a previous experiment, unexpectedly supported a spontaneous adoptive IgE anti-OA response.

Group A - 16 weeks of age, 9 weeks after immunization.
B - 12 weeks of age, 5 weeks after immunization.
X - 13 weeks of age
Y - 9 weeks of age.

Primed spleen cells (Groups A,B) were suspended either in Medium 199 alone or in Medium 199 containing 20% supportive FCS for injection into irradiated recipients (X or Y).

response. Immunized mice were bled immediately before being sacrificed to remove their spleens. During the preparation of the primed cell suspension, the blood was allowed to clot at 37°C. Serum was separated by centrifugation and was held on ice until required. Control sera from unimmunized mice were obtained in the same manner. Figure 27 shows that 0.2 ml of serum taken from donor mice on the day of transfer, when injected intravenously into irradiated recipients immediately before transfer of primed cells, reduced the adoptive IgE anti-OA titres by more than 90%. Suppression was equally effective whether the antibody was injected immediately before primed cells or whether cells and antibody were mixed in vitro and injected together.

These results were reproduced using anti-OA sera, which had been produced in BDF1 mice and NZW rabbits by the injection of OA in FCA. PCA assays showed that these sera contained no appreciable IgE antibody to OA. Therefore any demonstration of suppressive activity could be attributed to immunoglobulins of other classes. That the suppressive capacity was associated with immunoglobulins was demonstrated by repeating this experiment with gamma globulin fractions of both mouse and rabbit antisera to OA.

To facilitate the further study of anti-OA on the adoptive IgE anti-OA response, an attempt was made to raise relatively large volumes of mouse antiserum. These efforts met with little success. Repeated injections of BDF1 mice with 10 μg of OA in FCA, subcutaneously, produced serum

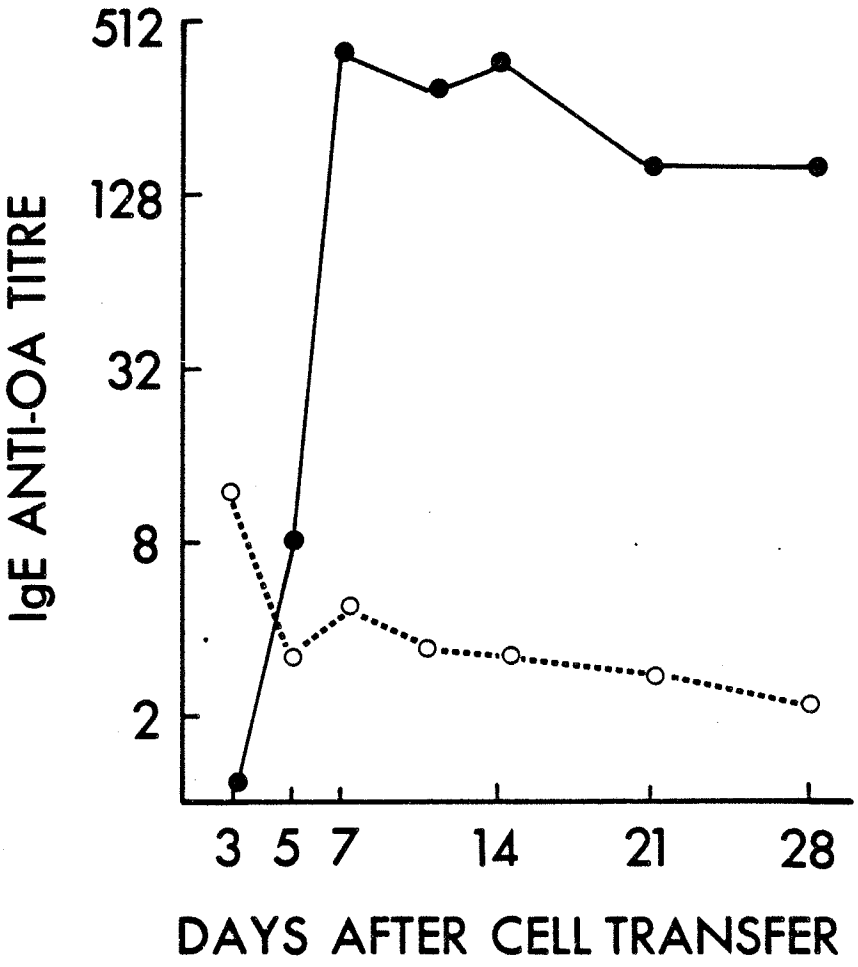


Figure 27.

The effect of donor serum on the adoptive IgE anti-OA response. Groups of six irradiated mice were injected i.v. with either 0.2 ml of normal serum (●) or 0.2 ml of serum taken from donors of primed cells on the day of transfer (o). Immediately after the injection of serum, $5 \times 10^7$ OA-primed spleen cells were injected into irradiated recipients.

hemagglutination titres of 1024 at most. Further intraperitoneal injections of OA in FCA boosted the titre to 4096 and resulted in the production of relatively large volumes of ascitic fluid with comparable anti-OA titres. Unfortunately, the ascitic fluid obtained clotted repeatedly when attempts were made to purify it. Despite attempts to induce formation of a final clot by the addition of calcium chloride (36), the globulin fraction obtained by precipitation of the material with an equal volume of saturated ammonium sulphate clotted when dialyzed or when thawed. Moreover, removal of clotted material by centrifugation at 100,000g for 1 hour immediately before injection into mice did not alleviate the toxic effects of the material which killed almost half of the experimental mice within a few minutes of injection. This material was, therefore, judged unsuitable for use. The addition of 100 units of heparin per ml did not alleviate this problem.

Rabbit antiserum to OA, however, was available in relatively large volumes and, on handling, did not present the problems seen with mouse anti-OA from ascitic fluid. To determine whether the Fc portion of antibody was required to effect suppression of the adoptive anti-OA response, the capacity of $F(ab')_2$ anti-OA to inhibit the transferred response was assessed. $F(ab')_2$ fragments of rabbit anti-OA or of normal rabbit immunoglobulin were prepared by pepsin digestion of the immunoglobulin fractions eluted from a 10 ml column of protein A-Sepharose. Five ml volumes of serum

which, on the average yielded 23.5 mg of immunoglobulins, were processed in this way to provide the fraction designated as intact immunoglobulin. An aliquot of intact immunoglobulin was then digested with 3% (w/w) pepsin and the neutralized digest was reapplied to the Protein A-Sepharose column to recover material which no longer bound to the column but which, in the case of anti-OA, still cross-linked RBC's to which OA had been coupled. This material was designated the $F(ab')_2$ fragment of the immunoglobulin preparations. For every 5.0 ml of starting serum an average of 5.8 mg of $F(ab')_2$ fragments were recovered. The hemagglutinating antibody titre of intact anti-OA immunoglobulin at a concentration of 10 mg/ml was $1 \times 2^{14}$ and of the $F(ab')_2$ fragments at the same concentration was $1 \times 2^{15}$.

Aliquots of a suspension of OA-primed spleen cells were supplemented with decremental amounts of normal rabbit immunoglobulin, $F(ab')_2$ fragments of normal rabbit immunoglobulins, intact rabbit anti-OA immunoglobulin or with $F(ab')_2$ fragments of rabbit anti-OA immunoglobulin and injected into irradiated recipients. Table 14 indicates that intact normal rabbit immunoglobulin had no effect on the adoptive IgE anti-OA response. $F(ab')_2$ fragments of normal immunoglobulin produced a transient (day 6) suppression when large quantities of material were injected. All quantities of intact anti-OA tested completely suppressed the adoptively transferred anti-OA response. In contrast, $F(ab')_2$ fragments of this same antibody produced only a transient (day 6 and day

TABLE 14

EFFECT OF $F(ab')_2$ ANTI-OA ON THE ADOPTIVE RESPONSE

| Antibody (a*) | Amount per Recipient mg | Adoptive IgE anti-OA titre (b*) Day 6 | Day 10 | Day 19 |
|---|---|---|---|---|
| - | - | 512 | 1024 | 256 |
| Normal rabbit Ig | 10.0 | 512 | 800 | 256 |
| | 5.0 | 512 | 512 | 256 |
| Normal rabbit F(ab')2 | 5.0 | 32 | 256 | 256 |
| | 0.5 | 1024 | 512 | 512 |
| Intact anti-OA | 5.0 | 0 | 0 | 0 |
| | 2.5 | 0 | 0 | 0 |
| | 1.25 | 0 | 0 | 0 |
| | 0.65 | 0 | 0 | 0 |
| | 0.312 | 0 | 0 | 0 |
| | 0.156 | 0 | 0 | 0 |
| F(ab')2 anti-OA | 5.0 | 0 | 32 | 50 |
| | 2.5 | 0 | 80 | 256 |
| | 1.25 | 16 | 80 | 200 |
| | 0.65 | 50 | 256 | 256 |
| | 0.312 | 64 | 256 | 256 |
| Spleen cells in Medium 199 without FCS: | | <4 | <4 | <4 |

a*
$F(ab')_2$ fragments of globulin preparations of normal rabbit serum and of rabbit antiserum to OA were prepared as described in Materials and Methods.

b*
Primed spleen cells were obtained from mice immunized 5 weeks earlier with 1 µg OA in $Al(OH)_3$ i.p. Suspensions were prepared in Medium 199 containing 20% supportive FCS, supplemented with the antibody preparations as indicated, and injected into irradiated recipients. Results show the IgE anti-OA titres in the sera of recipients 6, 10 and 19 days after transfer of cells.

10) suppression when 5.0, 2.5 or 1.25 mg of antibody preparation was injected per recipient. By day 19 after transfer, the IgE anti-OA titres in recipients of all but the largest amounts of $F(ab')_2$ fragments of anti-OA had reached control unsuppressed levels. On comparing the complete suppression seen with 156 ug of intact Ig to the partial suppression seen with 5,000 ug of $F(ab')_2$ fragments, it is possible to estimate that the intact immunoglobulin is at least 20 times (5000/156) x (1/1.6) more efficient in suppressing the adoptively transferred IgE anti-OA response. The factor is derived from the assumption that $F(ab')_2$ dimers (M.W.=100,000) contain approximately 1.6 times the number of antigen binding sites as does an equivalent amount of intact immunoglobulin (M.W.=165,000).

The objective was to develop a system in which the effects of sequential delivery of stimulatory (FCS) and inhibitory (antibody) stimuli on the primed spleen cell population could be analyzed. It had already been demonstrated (Table 5) that an in vitro pulse of FCS to primed spleen cells was sufficient to initiate the production of antibody in irradiated recipients of those cells. Experiments were carried out in which primed spleen cells suspended in Medium 199 supplemented with 20% FCS were exposed to anti-OA serum in vitro for a period of 60 - 90 minutes before being washed and injected into irradiated recipients. Several difficulties were encountered using this approach. Homologous antisera, obtained from BDF1 mice by immunization

with OA in FCA or in $Al(OH)_3$ were toxic to mouse spleen cells, killing 25 - 30% of lymphocytes during a one hour exposure. Normal mouse serum was equally toxic. The final concentration of mouse serum in such cultures ranged from 5 to 20% and corresponded to the amount of antibody which maximally suppressed the adoptive anti-OA response when injected into irradiated recipients at the time of cell transfer. Because of the cell death during incubation cell concentrations were adjusted so that equal numbers of live cells were injected into different groups of irradiated recipients. Nevertheless, no significant difference could be detected between the effects of in vitro exposure to anti-OA and to control sera on the adoptive anti-OA response (Figure 28).

Rabbit anti-OA was used in similar experiments. This material was not toxic to mouse spleen cells. As shown in Figure 29, however, the suppressive capacity of anti-OA was much less effective when the cells were exposed to the antibody in vitro than when the anti-OA was injected directly into irradiated recipients before transfer of primed spleen cells.

These experiments had been carried out at $37^{\circ}$ C because of the requirement of physiological temperatures for the binding of the Fc portion of immunoglobulins to murine Fc receptors on cell membranes. The possibility existed that some cell membrane component essential for the induction of specific suppression, such as complexes of OA and anti-OA, was

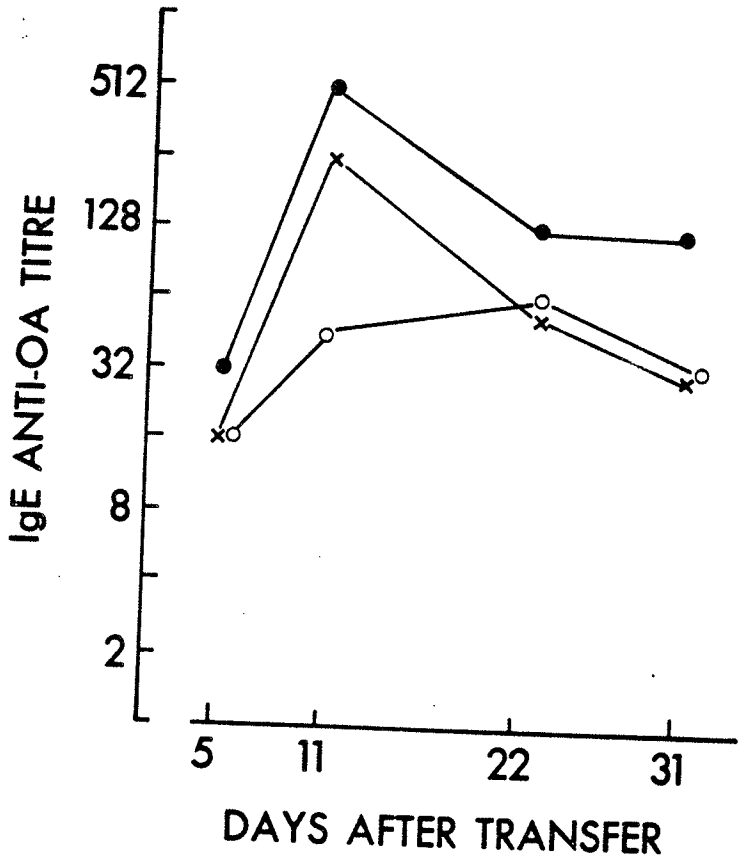


Figure 28.

In vitro exposure of OA-primed cells to mouse anti-OA. Primed spleen cells were incubated at 37°C for 60 min. in Medium 199-20% FCS (•), in Medium 199-20% FCS + mouse anti-OA serum (x) or in Medium 199-20% FCS + normal mouse serum (o). Cells were exposed in vitro to 0.2 ml serum per 5 x 10 cells. Cells were washed and resuspended in Medium 199-20% FCS before transfer to irradiated recipients.

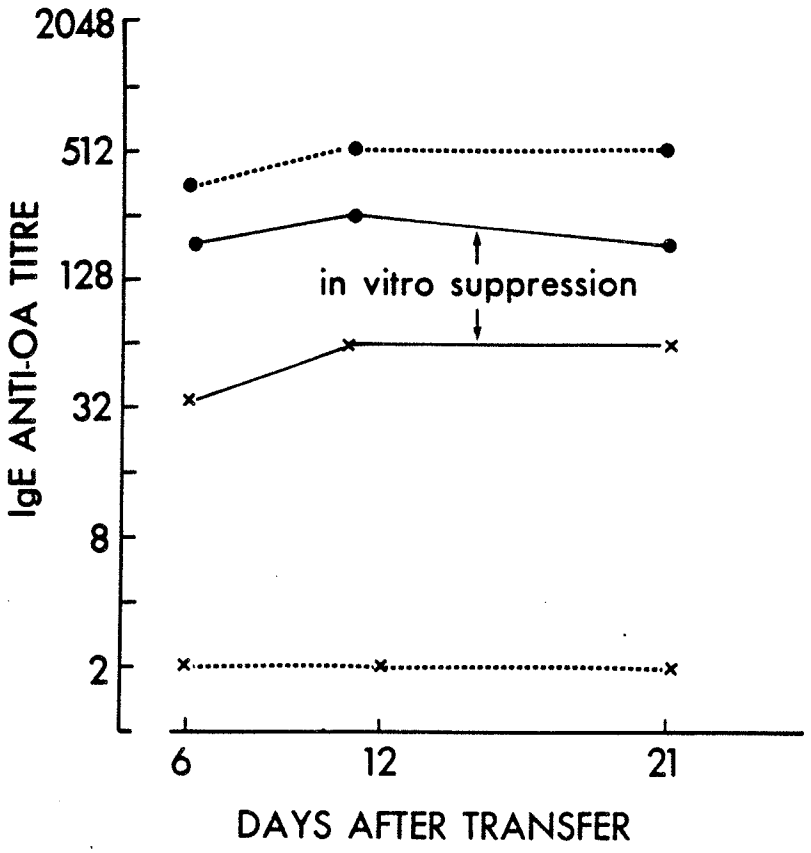


Figure 29.

A comparison of the effects of in vitro and in vivo exposure of OA-primed cells to rabbit anti-OA. Primed spleen cells were prepared in Medium 199. In vitro (——) suppression was attempted by incubating, at 37° C for 60 min., aliquots of the cell suspension with rabbit anti-OA serum (x) or with normal rabbit serum (•) to a concentration of 0.2 ml of serum per $5 \times 10^7$ cells. Cells were washed in Medium 199 and resuspended in medium containing 20% supportive FCS for injection into irradiated recipients. As an in vivo (....) control for the effectiveness of the rabbit sera used, 0.2 ml of rabbit anti-OA or of normal rabbit serum was injected i.v. into two groups of irradiated mice immediately before transfer of $5 \times 10^7$ primed cells in medium containing 20% supportive FCS.

removed by capping at 37°C. For this reason the experiments were repeated with all steps for the procedure being carried out in an ice-bath. In vitro exposure to anti-OA remained ineffective under these conditions.

Experiments were also carried out in which primed cells were exposed to antibody in vitro, washed, exposed to FCS in vitro, washed again, suspended in Medium 199 and injected into irradiated recipients. Under these conditions, the suppression of the adoptive IgE anti-OA response was again minimal. Evidently short-term exposure to anti-OA in vitro could not mimic the suppressive effects of the same antibody when injected into the irradiated recipient at the time of cell transfer.

The ability of anti-OA to suppress the adoptive IgE anti-OA response, under in vivo conditions, suggested that antigen was present somewhere in the system. Since spleen cells suspensions were washed before exposure to antibody in the irradiated recipients, it seemed most likely that any remaining antigen would be cell bound and most likely accessible on a cell membrane. The possibility that antigen was present in the fetal calf serum used to prepare the donor cell suspension was discounted by the observation that the adoptive IgE anti-OA response generated by cells prepared in Medium 199 alone was also completely suppressed by injecting the irradiated recipient with anti-OA immediately before transferring primed spleen cells (Figure 30).

There followed a series of experiments aimed

primarily at identifying the cell type carrying the stimulus, supposedly antigen, that was critical to the development of the adoptive IgE anti-OA response. These studies involved depletion, from the primed spleen cell suspension, of cell types known to be involved in the development of humoral immune responses.

## 3.7 Cellular components of the adoptive IgE anti-OA response

### 3.7.1 Macrophage independence

Experiments were carried out to assess the contribution of donor macrophages to the transferred response. Since macrophages have been shown to phagocytose antigen and to play a role in the processing and presentation of antigen to lymphocytes during an immune response (138), the ability of macrophage-depleted primed spleen cells to support the adoptive anti-OA response was examined.

Because of the difficulty that exists when trying to insure complete depletion of macrophages three different procedures were used to remove these cells. It was assumed that such an approach would assure a reliable assessment of the contribution of macrophages to the adoptive transfer model under investigation.

The first method employed to remove macrophages was density gradient centrifugation. A suspension of primed spleen cells was applied to a solution of Ficoll-Metrizoate (density=1.07) and centrifuged at 400 g for 20 minutes at room

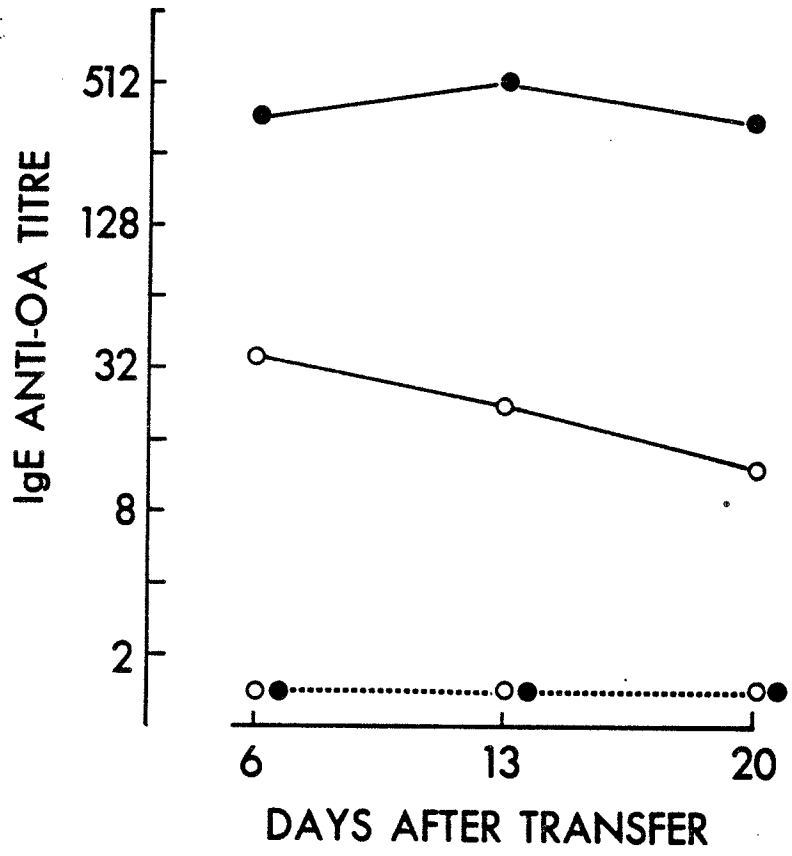


Figure 30.

Suppression of the FCS-independent adoptive IgE anti-OA response by antibody to OA. Irradiated recipients were injected with 0.3 ml of a globulin fraction of mouse anti-OA serum (....) or with 0.3 ml of normal mouse immunoglobulin (——) before receiving $5 \times 10^7$ primed spleen cells suspended in Medium 199 (o) or in Medium 199 supplemented with 20% supportive FCS (o).

temperature. Under these conditions, all the red cells and 90% of the white cells in the suspension passed through the Ficoll-Metrizoate to form a pellet. The remaining 10% of the white cells were recovered at the interface of the medium and the Ficoll-Metrizoate layer. Staining with Giemsa and May-Grunwald stains revealed a population of homogeneously small cells. More than 98% of these cells appeared to be lymphocytes as judged by the ratio of nuclear to cytoplasmic area and by the pale, agranular staining of the cytoplasm. One to two percent of the cells had the characteristics of eosinophils, i.e. multi-lobed nuclei and a cytoplasm packed with bright purple-orange granules. The slightly larger cell with a U-shaped nucleus which had been identified in the starting cell preparation as a macrophage was not seen in the recovered population of small lymphocytes.

Despite the low recovery (less than 10%) of cells after depletion of macrophages, these small lymphocytes were capable of supporting an adoptive IgE anti-OA response of the same magnitude as that generated by an equivalent number of unfractionated primed spleen cells (Table 15, Expts. 1 and 2).

Macrophages were also removed from suspensions of primed spleen cells using two different additional methods based on the tendency of these cells to adhere to solid surfaces. A suspension of primed spleen cells was divided into three aliquots. One aliquot was held on ice as the unfractionated cell suspension. The remaining cells were either passed through a column of Sephadex G-10 supported on a layer of

TABLE 15

EFFECT OF MACROPHAGE DEPLETION ON THE ADOPTIVE IgE ANTI-OA RESPONSE

| Expt. | Method of Depletion | Yield % | IgE anti-OA titre | |
|---|---|---|---|---|
| | | | Unfractionated | Depleted |
| 1 | Density gradient | 5.8 | 800 | 1024 |
| 2 | Density gradient | 9.0 | 512 | 1024 |
| 3 | Column of glass beads and G-10 | 10.0 | 400 | 845 |
| 4 | Petri dishes | 16.0 | 400 | 480 |
| 5 | Columns of glass beads and G-10 | — | 1320 | 1320 |
| 6 | Petri dishes | — | 1320 | 1320 |

Primed spleen cells were obtained from mice immunized 6 - 10 weeks earlier with 1 μg OA in $Al(OH)_3$ i.p.

Fractionation of cells was carried out as described in Materials and Methods. For injection into irradiated recipients, all cell preparations were supplemented with 20% supportive FCS.

$$\text{Yield} = \frac{\text{No. of cells recovered after depletion}}{\text{No. of cells in starting preparation}} \times 100$$

glass beads or were layered onto glass Petri dishes. Both procedures were carried out at 37°C. Cells which after 90 minutes had not adhered to Petri dishes were layered into fresh Petri dishes for a second cycle of adherence.

The recovered non-adherent cell populations were washed and suspended in Medium 199-20% FCS for injection into irradiated recipients. Results of experiments 3 and 4 recorded in Table 15 indicate that removal of adherent cells from the suspension of primed spleen cells did not affect the ability of the lymphocytes to produce IgE anti-OA in irradiated recipients.

### 3.7.2 T cell dependence

Having established that macrophages probably did not play a primary role in the initiation of the adoptive anti-OA response attention turned to the role of donor T cells in the experimental model. Kontiainen and Mitchison (133,139), working with an in vitro system had suggested that the antigen responsible for continued antibody production was found, complexed to antibody, on the plasma membranes of T cells. To determine the effect of T cell depletion on the adoptive IgE anti-OA response primed spleen cells were treated with rabbit antiserum to mouse brain-associated Thy-1 plus complement. This antiserum killed 23 - 26% of spleen cells in cytotoxicity assays. The specificity of this antiserum for T cells was demonstrated by its ability to eliminate the in vitro responsiveness of normal mouse spleen cells to the T

cell mitogen, Concanavalin A, while leaving the mitogenic response to the B cell mitogen, lipopolysaccharide, unaffected (Table 16). Treatment of OA-primed spleen cells with rabbit anti-mouse brain associated Thy 1 and complement before transfer to irradiated recipients completely prevented the adoptive IgE anti-OA response (Figure 31). Treatment of an aliquot of the same starting spleen cell preparation with normal rabbit serum and complement did not affect the adoptive IgE anti-OA response.

When these experiments were repeated using commercially available mouse monoclonal antibody, clone F7D5, anti-Thy 1.2 and complement to remove T cells, the adoptive IgE anti-OA response was again shown to be completely T cell dependent. In cytotoxicity assays the mouse monoclonal anti-Thy 1.2 plus C killed 12 to 18% of spleen cells as assessed by trypan blue exclusion. Despite the relatively low percentage of cells killed the ability of this monoclonal antibody to remove functional T cells was demonstrated in an independent plaque forming cell assay for which data will be presented in section 3.7.4.

The T cell dependence of adoptive IgE anti-OA production was also demonstrated in experiments where production of antibody by recipient mice was not dependent on FCS (Figure 32). To investigate the nature of the interaction of B and T cells that led to adoptive IgE anti-OA production purified suspensions of B and T cells were prepared from the spleens of donor mice. Because the methods of B and

TABLE 16

THE EFFECT OF T CELL DEPLETION ON THE MITOGEN RESPONSIVENESS OF NORMAL BDF1 SPLEEN CELLS

| Treatment | 3-H Thymidine Uptake cpm | | |
|---|---|---|---|
| | Medium | LPS | Con A |
| C | 5,338 | 61,369 | 178,158 |
| Rabbit anti-mouse brain-associated Thy.1 + C | 1,356 | 74,967 | 2,390 |

Spleen cells were treated with either NGPS (C) or with rabbit antiserum to mouse brain-associated Thy.1 + C, washed and cultured at 6 x $10^5$ viable cells/culture in RPMI 1640 containing 5% FCS. Cultures were supplemented with medium alone, with LPS at 50 µg/ml or with Con A at 5 µg/ml. 3-H Thymidine was added for the final 18 hours of a 72-hour culture. The cells were harvested using a Skatron automatic harvester and were assayed for thymidine incorporation.

(Cells were cultured by J.Wilkins.)

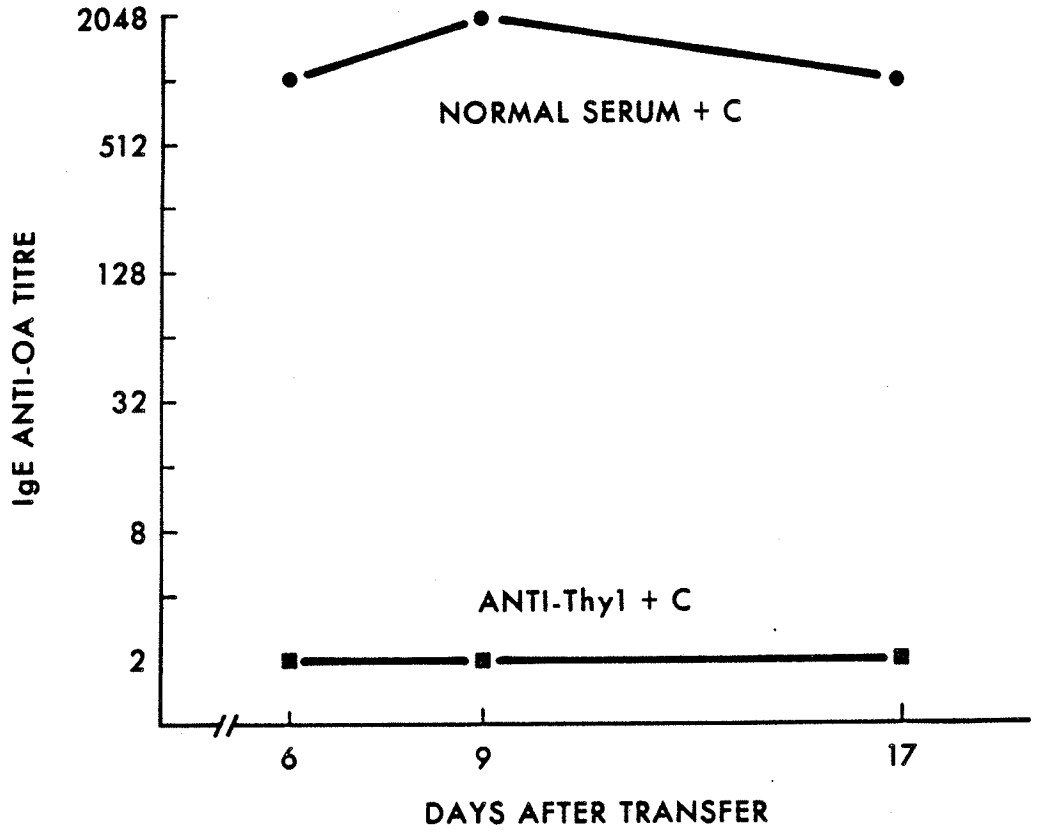


Figure 31.

The effect of T cell depletion on the adoptive IgE anti-OA response. OA-primed spleen cells were treated with normal rabbit serum + C (●) or with rabbit anti-mouse brain-associated Thy.1 + C (■). Dead cells were removed by passage through a Ficoll-Metrizoate gradient before being washed and resuspended in Medium 199 containing 20% supportive FCS. Each of the irradiated recipients in the two experimental groups were injected with $5 \times 10^7$ viable cells.

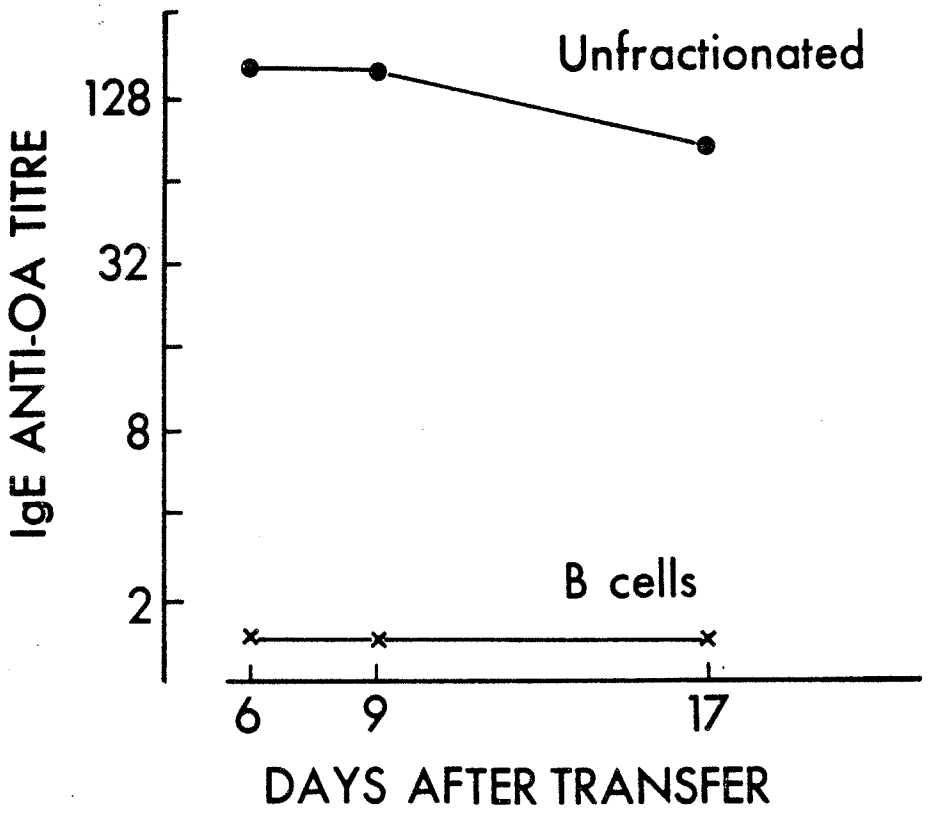


Figure 32.

The T cell dependence of the FCS-independent adoptive response. OA-primed spleen cells were suspended in Medium 199 and either injected directly into irradiated recipients (•) or depleted of T cells (x) by reaction with rabbit anti-mouse brain-associated Thy.1 + C before injection. Irradiated recipients received $5 \times 10^7$ unfractionated cells or $3 \times 10^7$ B cells.

T cell isolation employed had not been in routine use in the laboratory it was necessary to validate the purification procedures by characterizing the isolated cell populations. The following section, 3.7.3, contains data obtained on the plasma membrane markers and functional capacities of isolated B and T cells. The results of investigation into the interaction of these cell populations in the adoptive IgE anti-OA response will be presented in section 3.7.4.

### 3.7.3 Characterization of isolated T and B cells

Two methods were employed to obtain T cells. B cells were eliminated from a suspension of spleen cells either by treatment with rabbit anti-mouse Ig + C or by passage through columns of nylon wool. Initially, treatment with cytotoxic antiserum was favoured because it appeared to offer a more specific method for the removal of B cells. Moreover the manipulations involved in this procedure were identical to those used for the removal of T cells, only the specificity of the antisera differed. As a result the simultaneous preparation of B and T cells would have been facilitated.

The removal of B cells by treatment with cytotoxic anti-Ig + C was adapted from the method used by Yuan and Vitetta (122) to remove isotype specific cells from a variety of cell suspensions. This method required the addition of 0.01 M sodium azide to the cell suspension. To establish the conditions for maximal specific killing of B cells, a suspension of cells was treated with decreasing concentrations

of antibody. As shown in Figure 33, this produced a parabolic cytotoxicity curve due to inefficient killing by high concentrations of antibody. This pattern was seen with two different preparations of antibody and may be a characteristic of this method. Figure 33 shows also that the addition of azide increased the net cytotoxicity at all antibody concentrations by 10 - 15%. Rabbit anti-mouse Ig + C killed 53% (n=7) of spleen cells, 18% of bone marrow cells and less than 1% of thymus cells. Adsorption of this antiserum with $1 \times 10^8$ thymocytes per ml of antiserum did not reduce the cytotoxicity against spleen cells. Simultaneous treatment of spleen cells with optimal concentrations of anti-mouse IgG and anti-Thy 1.2 killed 81 - 86% of spleen cells. The additive effect of the two antisera indicated that they were specific for different populations of lymphoid cells.

T cells were obtained also by passing cell suspensions through columns of nylon wool, as described by Julius, Simpson and Herzenberg (126).

The surface characteristics of the isolated B and T cell populations were determined by treating the purified cell suspensions with anti-Ig + C and anti-Thy 1.2 + C. The presence of surface Ig (sIg(+)) on cells was also assessed by a rosetting assay in which lymphocytes were mixed with SRBC to which were coupled antibody to murine immunoglobulins. The results are shown in Table 17. The unfractionated cell population was shown to contain 28% T cells and about 55% B

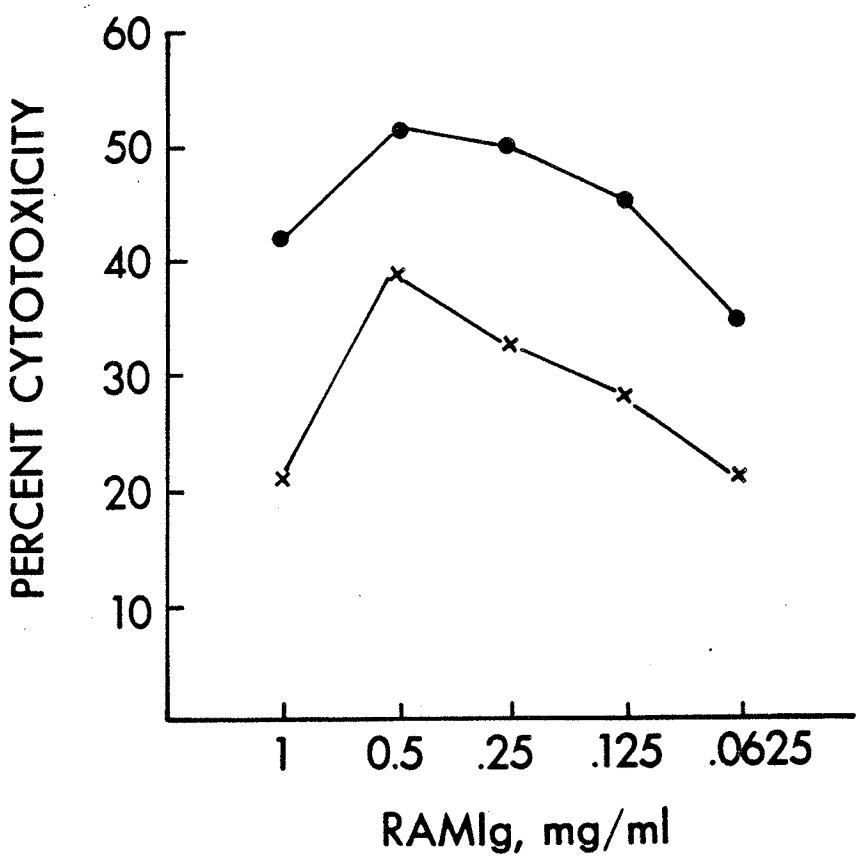


Figure 33.

Titration of RAMIg in a cytotoxicity assay. Spleen cells at a concentration of 1 x $10^7$/ml were reacted with decreasing concentrations of a globulin preparation of rabbit anti-mouse Ig in the absence (x) and presence (•) of 0.01 M $NaN_3$ for 30 min. at 0° C. NGPS (C) was added to the suspensions which were then transferred to a 37° C water bath for 45 min. Viability was assessed by exclusion of trypan blue dye at a final concentration of 0.1%. Non-specific killing, determined by incubating cells with antibody or C alone, was subtracted from the total number of dead cells to obtain the data illustrated.

cells. Therefore, using the methods described about 15% of splenic lymphocytes carried neither the major T cell nor the major B cell membrane markers.

T cells prepared by exposure of spleen cells to anti-Ig + C contained a small number of cells with surface Ig as detected by both the cytotoxicity assay and the rosetting assay. Moreover, only 69% of the putatively purified T cell preparation would be identified as such by cytotoxic anti-Thy 1 antibody. As a result almost 25% of the lymphocytes in the T cell preparation could not be identified as T or B cells.

T cells recovered from nylon wool columns contained a slightly higher (76%) proportion of identifiable T cells. However, a discrepancy arose when testing the nylon wool purified cells for B cell contamination. Anti-Ig + C revealed a 12% contamination by B cells while the rosetting assay could identify only 2% of the population as B cells. Treatment of spleen cells with anti-Thy 1 + C successfully eliminated all T cells as judged by re-assay with the same antiserum. Approximately 75% of this putative B cell population was shown to carry surface immunoglobulin as detected by both the rosetting assay and the cytotoxic anti-Ig + C.

Experiments were carried out in which OA-primed spleen cells were depleted of B cells by anti-Ig + C before transfer of the surviving (T cell) population into irradiated recipients. These isolations were carried out with a view to stimulating or inhibiting independently B and/or T cells

TABLE 17

SURFACE Ig AND Thy 1.2 OF PURIFIED B AND T CELLS

| Cell population | %Ig (+) as Assessed by: | | % Anti-Thy 1 (+) |
|---|---|---|---|
| | RAMIg + C | Rosetting | (b*) |
| Unfractionated | 53 | 58 | 28 |
| T (anti-Ig + C) (a*) | 8 | 6 | 69 |
| T (nylon wool passed) | 12 | 2 | 76 |
| B (anti-Thy 1 + C) | 78 | 76 | 2 |

a*
B and T cell populations were isolated by the methods indicated in brackets. Cytotoxicity and rosetting assays were carried out as detailed in Materials and Methods.

b*
The antiserum used to assess Thy 1(+) cells was rabbit anti-mouse brain-associated Thy.1 antigen.

before recombination of the two populations for injection into irradiated recipients. Unexpectedly, in two out of three experiments, the putative T cell population obtained by treating primed spleen cells with anti-Ig + C supported an adoptive IgE anti-OA response that was almost equivalent to that generated by the unfractionated population (data not shown). Such a sizable antibody response was being supported by a population of cells from which almost 90% of the B cells, as defined by the presence of surface immunoglobulin, had been removed. An independent assay was, therefore, carried out to determine whether the depletion indicated by the disappearance of surface Ig was paralleled by a reduction in antibody forming capacity of the same cell population.

Spleen cells were taken from mice which had been immunized three weeks earlier with $5 \times 10^8$ SRBC. Aliquots of a single suspension of primed cells were fractionated by cytotoxicity with anti-Ig + C or anti-Thy 1.2 + C, or by passage through columns of nylon wool. The resulting populations alone or after recombination were injected into irradiated (600 Rads) mice which were then immediately injected intravenously with $5 \times 10^8$ SRBC. Six days after the transfer, the spleens of the irradiated recipients were removed and assayed for their content of plaque forming cells against SRBC. Table 18 shows that treatment of primed spleen cells with anti-Thy 1.2 + C prevented the development of IgG secreting cells in this T cell dependent response. However, in both experiments recorded, treatment of cells with anti-Ig

+ C permitted the generation of an adoptively transferred response which is greater than half that seen with unfractionated cells. T cells obtained by passage through columns of nylon wool supported a response equal to 17% of the control.

Table 19 shows that, in all cases, the addition of T cells to the purified B cell population facilitated an antibody response comparable to or greater than that generated by unfractionated cells.

These experiments showed that treatment of spleen cells with anti-Ig + C either eliminated only a few B cells as judged by the ability of the surviving cells to generate antibody secreting cells in an irradiated recipient animal or the surviving cells had an unusually high capacity for antibody production in the same system. This was true both for the adoptive IgE anti-OA response and the IgG SRBC response. Thus, the inadequacy of this method of B cell elimination was not revealed by the detection of membrane markers on spleen cells. These observations have since been confirmed by Mond et al (140) with respect to the IgG response.

As a result of these observations T cells for further experiments were obtained by passage of spleen cells through columns of nylon wool while B cells were obtained by treatment of an aliquot of the same starting cell suspension with anti-Thy 1.2 + C.

TABLE 18

EFFECTIVENESS OF CELL PURIFICATION AS ASSESSED BY PFC ASSAY (a*)

| Expt. (b*) | Treatment | Cell Population | No. Cells Injected | IgG PFC/Spleen (c*) % of Unfractionated |
|---|---|---|---|---|
| 1 | Nil | Unfractionated | $6 \times 10^6$ | 100 (112,000) (d*) |
| | Anti-Thy 1 + C | B | $4 \times 10^6$ | 3 |
| | Anti-Ig + C | T | $2 \times 10^6$ | 55 |
| | Nylon wool | T | $2 \times 10^6$ | 17 |
| 2 | Nil | Unfractionated | $6 \times 10^6$ | 100 (19,500) |
| | Anti-Thy 1 + C | B | $4 \times 10^6$ | <1 |
| | Anti-Ig + C | T | $2 \times 10^6$ | 54 |
| | Nylon wool | T | $2 \times 10^6$ | 17 |

a*
SRBC-primed spleen cells were fractionated by the methods indicated and the resulting populations were injected into irradiated (600 R) BDF1 mice followed by challenge with $5 \times 10^8$ SRBC i.v. Six days later the spleens of recipients were assayed for anti-SRBC PFC.

b*
Donor spleen cells were obtained 7 days after priming with $5 \times 10^8$ SRBC for Expt. 1 and 20 days after priming for Expt. 2.

c*
IgM PFC were less than 100 per spleen in all cases.

d*
Brackets contain the number of PFC per spleen.

TABLE 19

GENERATION OF PFC BY RECOMBINED, PURIFIED B AND T CELLS

| Expt. | Donor cell (a*) Population | No. Cells Injected | IgG PFC/Spleen (b*) % of Unfractionated |
|---|---|---|---|
| 1 | Unfractionated | $6 \times 10^6$ | 100 |
| | B cells + T cells (anti-Ig + C) | $4 \times 10^6$ + $2 \times 10^6$ | 81 |
| | B cells + T (nylon wool) | $4 \times 10^6$ + $2 \times 10^6$ | 177 |
| 2 | Unfractionated | $6 \times 10^6$ | 100 |
| | B cells + T (anti-Ig + C) | $4 \times 10^6$ + $2 \times 10^6$ | 200 |
| | B cells + T (nylon wool) | $4 \times 10^6$ + $2 \times 10^6$ | 138 |

a*
Cell populations are those described in Table 17. All were obtained from a pool of SRBC-primed spleen cells. B cells were isolated by treatment of the starting spleen cell suspension with anti-Thy 1.2 + C. T cells were prepared by the methods indicated in brackets. When required, B and T cells were mixed in vitro before injection into irradiated recipients.

b*
Immediately after transfer, recipients were challenged with $5 \times 10^7$ SRBC i.v. Six days later the spleens of recipients were assayed for anti-SRBC PFC.

3.7.4 Proliferation of B and T cells

B and T cells, isolated as indicated above, were prepared from the spleens of mice immunized 6 weeks earlier with 1 μg OA in $Al(OH)_3$ i.p. Recombination of the two purified cell populations before injection into irradiated recipients allowed demonstration of the interaction necessary between B and T cells for the generation of the adoptive IgE anti-OA response (Figure 34). As seen earlier, B cells alone were unable to support the IgE anti-OA response in irradiated recipients. The small response supported by T cells was consistently observed but amounted to only approximately 2% of the IgE anti-OA titre supported by unfractionated cells. Recombination of the purified B and T cell population led to the production of IgE anti-OA titres equivalent to, or slightly greater than, those produced by unfractionated cells. The increment of IgE anti-OA titres produced by recombined B and T cells over those generated by unfractionated cells was always observed but never exceeded a 2- to 3-fold increase of the response.

Experiments described so far indicated that the adoptive IgE anti-OA response was macrophage independent and T cell dependent. Earlier data on the effect of treating donor cells with mitomycin C had established that cellular proliferation was required for the development of the adoptive response. Since helper T cells from primed animals have been shown to exert their effect without needing to proliferate (141,143) and since there is evidence for the ability of

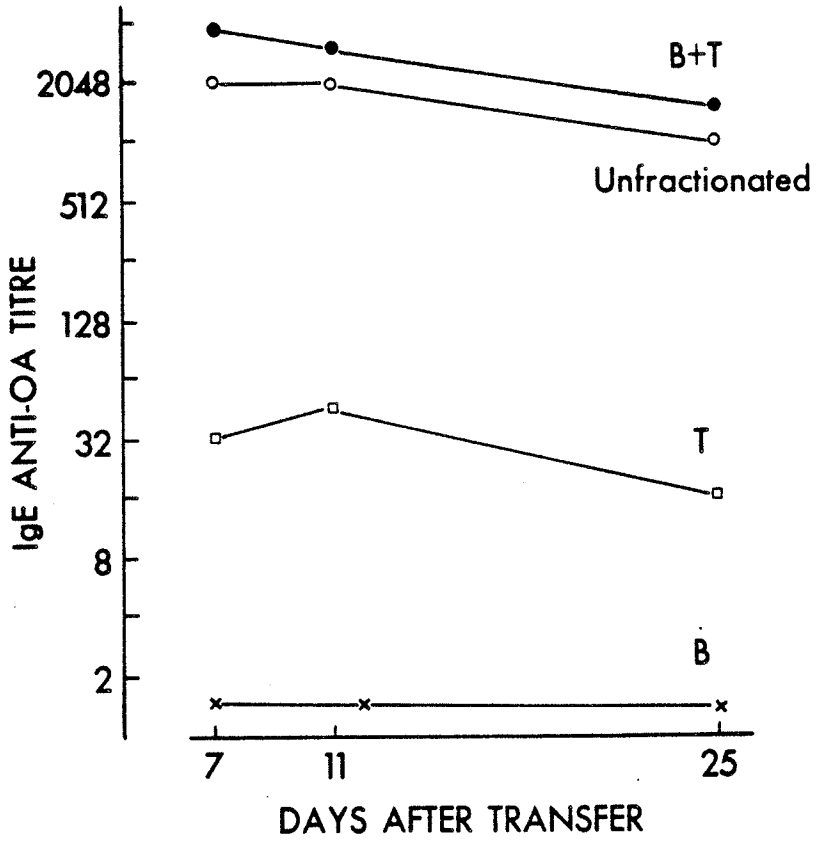


Figure 34.

B and T cell cooperation in the generation of an adoptive IgE anti-OA response. B cells were obtained by treatment of OA-primed cells with mouse monoclonal anti-Thy 1.2 + C. T cells were obtained by passage through nylon wool of an aliquot of the same starting cell suspension. All cell populations were suspended in Medium 199-20% FCS for injection into irradiated recipients according to the following protocol: $3 \times 10^7$ unfractionated cells (o), $2 \times 10^7$ B cells (x), $1 \times 10^7$ T cells (□), $2 \times 10^7$ B cells + $1 \times 10^7$ T cells (■).

memory B cells to become antibody secretors without dividing (144,145), the following experiments were designed to establish whether replication of both B and T cells was required to effect IgE anti-OA production in irradiated recipients.

Experiments were carried out in which purified B and T cells were independently exposed to the mitotic inhibitor, mitomycin C, or to medium alone before being recombined, exposed to FCS and injected into irradiated recipients. Table 20 shows that treatment of unfractionated cells with mitomycin C abolished the adoptive IgE anti-OA response. Similarly, the independent exposure of purified B and T cells to mitomycin C prevented the recombined cell population from giving a transferred response. Untreated B cells, when mixed with mitomycin C treated T cells, produced an adoptive IgE anti-OA titre of 32, which is 12% of that produced by unfractionated cells and less than 5% of that generated by untreated, recombined B and T cells.

Similarly, mitomycin C treated B cells plus untreated T cells produced an adoptive IgE anti-OA response equivalent to only 11% of that produced by the recombined B and T cell population. These data indicated that both T and B cells must proliferate in order to successfully cooperate for the production of the adoptive IgE anti-OA response. One would expect that, if proliferation of both B and T cells were required for the maintenance of long term antibody production, then prevention of in vivo cell division should abolish an

TABLE 20

THE EFFECT OF INDEPENDENT EXPOSURE OF B AND T CELLS TO MITOMYCIN C ON THE ADOPTIVE IgE ANTI-OA RESPONSE

| Cell population (a*) | Adoptive IgE anti-OA titres Day 11 after transfer |
|---|---|
| Unfractionated | 256 |
| Unfractionated (mito) (b*) | 0 |
| B cells (c*) | 0 |
| T cells (d*) | 50 |
| B + T | 700 |
| B (mito) + T (mito) | 0 |
| B + T (mito) | 32 |
| B (mito) + T | 80 |

a*
The starting cell population was obtained from the spleens of mice that had been immunized with 1 μg OA in $Al(OH)_3$ i.p. six weeks earlier.

b*
Cells were exposed to Mitomycin C (mito) at a concentration of 40 μg/5 x $10^7$ cells/ml for 30 minutes at 37°C. Cells not exposed to Mitomycin C were similarly incubated but in Medium 199 only. Following incubation, all cell preparations were washed in Medium 199 and resuspenden in medium containing 20% supportive FCS before being injected into irradiated recipients.

c*
B cells were obtained by treating an aliquot of the starting spleen cell suspension with mouse monoclonal anti-Thy 1.2 + C.

d*
T cells were obtained by passage of an aliquot of the starting cell suspension through columns of nylon wool. Non-supportive FCS, Reheis M41512, was used in the medium during this fractionation of cells.

established antibody response. Radiation was used as the means for inhibiting mitosis in order to test this postulate in the live animal.

## 3.8 Radioresistance of established IgE antibody production

BDF1 mice which had been immunized eight weeks earlier with 1 μg OA in $Al(OH)_3$ i.p. were exposed to doses of irradiation ranging from 0 to 1,000 Rads. Animals exposed to 800 and 1,000 Rads were divided into two groups, one of which received $2 \times 10^7$ normal bone marrow cells to reduce mortality the other being untreated. IgE anti-OA titres in the sera of both groups of these immunized, treated mice were monitored for 32 days after irradiation. Table 21 indicates that irradiation doses of up to 1,000 Rads did not affect the circulating levels of IgE anti-OA in immunized mice.

Virtually identical data were obtained (data not shown) on the radioresistance of an established IgE response in adoptively immunized mice. Mice producing stable levels of IgE anti-OA antibody 8 weeks after adoptive transfer of cells were exposed to doses of irradiation up to 1,000 Rads. As in intact, conventionally immunized, animals IgE antibody levels in the adoptively immunized mice were unaffected by even the highest doses of irradiation. These results implied that there existed, in vivo, a population of cells whose activity was not dependent on proliferation but which, nevertheless, were capable of maintaining high, stable titres of IgE anti-OA months after immunization. To verify that

TABLE 21

THE EFFECT OF IRRADIATION OF IMMUNIZED MICE ON THE ESTABLISHED IgE ANTI-OA RESPONSE

| Irradiation dose(a*) Rads | $Log_2$ Anti-OA titre Days after irradiation | | | | |
|---|---|---|---|---|---|
| | 3 | 6 | 11 | 20 | 32 |
| 0 | 10 | 10 | 9.5 | 9.5 | 11.0 |
| 200 | 11 | 12 | 9.5 | 9.5 | 11.0 |
| 400 | 10 | 12 | 9.5 | 9.5 | 11.0 |
| 600 | 10 | 11 | 9.5 | 9.5 | 9.5 |
| 800 | 12 | 12 | 9.5 | 9.5 | 9.5 |
| 1000 | 12 | 11 | Died | - | - |
| 800 + 2 x $10^7$ BM (b*) | 11 | 11 | 9.5 | 9.5 | 8.0 |
| 1000 + 2 x $10^7$ BM | 12 | 12 | 11.6 | 9.5 | 9.5 |

a*
Groups of 4 mice were irradiated at the doses indicated 8 weeks after immunization with 1 μg OA in $Al(OH)_3$ i.p.

b*
Groups of mice receiving the highest doses of irradiation were reconstituted with 2 x $10^7$ normal bone marrow cells.

cell proliferation was necessary for the initiation of the adoptive response primed spleen cells obtained from mice 8 weeks after immunization were irradiated in vitro with different doses before being injected into irradiated recipients; the results are shown in Table 22. The magnitude of the adoptive IgE anti-OA response was reduced by 75% after exposure of primed spleen cells to 200 Rads. Exposure to 400 Rads reduced the antibody titres in recipients by more than 95%. Doses of 600 Rads or more completely abolished the adoptive IgE anti-OA response.

These results substantiated the conclusion that cellular proliferation was necessary to establish the adoptive response in recipient mice. Radiation resistant antibody secreting cells, if present in the suspension of primed spleen cells at the time of transfer, were either not present in numbers sufficient to contribute to the adoptive anti-OA response or were inhibited in the environment of the irradiated recipient. However, the ability of the established adoptive IgE anti-OA response to withstand exposure to 1,000 Rads suggested that the donor cell population contained the precursors capable of differentiating into a radiation resistant population. Such a radiation resistant population was evidently also present in intact, conventionally immunized mice.

TABLE 22

in vitro IRRADIATION OF OA - PRIMED CELLS (a*)

| Irradiation dose Rads | $Log_2$ anti-OA PCA titres Days after transfer | | | |
|---|---|---|---|---|
| | 6 | 11 | 20 | 32 |
| 0 (b*) | 10 | 10 | 9.5 | 11 |
| 200 | 8 | 7.6 | 7.6 | 7 |
| 400 | 4 | 3.0 | 3.8 | <2 |
| 600 | <2 | <2 | <2 | <2 |
| 800 | <2 | <2 | <2 | <2 |
| 1000 | <2 | <2 | <2 | <2 |

a*
Primed spleen cells were obtained from mice 8 weeks after immunization with 1 μg OA with $Al(OH)_3$ i.p. Suspensions were prepared in Medium 199. Aliquots of 2.5 ml containing $1 \times 10^7$ cells/ml were placed in 50 ml round bottomed polycarbonate tubes. After irradiation 0.5 ml of supportive FCS was added to each aliquot which was then divided equally among 4 irradiated recipients.

b*
A control group of immunized mice were not irradiated, but their IgE anti-OA titres were monitored at the same time as those of experimental groups.

# CHAPTER 4: DISCUSSION

## 4.1 Characteristics of the IgE anti-OA response in BDF1 mice.

Much has been learned over the past ten years about the regulation of IgE synthesis, particularly in mice and rats (146). In vitro studies of IgE synthesis have been difficult in all species even in experimental animals with augmented IgE synthesis or in humans with diseases characterized by greater than normal production. The basic problem in this regard is that the lymphoid cells which are accessible produce extremely small quantities of IgE antibodies. In face of these problems the thrust of the experiments described in this thesis was towards establishing, in experimental animals, a suitable model for studying IgE production in vivo; in particular the protracted nature of the response. A long term response was of particular interest because of its relevance to IgE production in allergen-sensitive persons. In such individuals IgE levels have been shown to increase steadily over a period of years (147). The important consequence of a rising IgE antibody level, from the point of view of the affected individual, is that it leads to an increased sensitivity to allergen. In vitro studies on histamine release from sensitized leucocytes (basophils) have shown that cells sensitized with serum taken from an allergic person when compared with cells sensitized with normal serum may be more than 1000 times more sensitive to allergen on the

basis of the antigen dose required to release 50% of the releasable histamine (148).

Persisting serum levels of specific IgE antibodies must be determined by a complex function involving both catabolic elimination of circulating IgE and addition of newly synthesized IgE. Newly synthesized IgE antibodies would originate, according to conventional thinking, from relatively short lived plasma cells which differentiated from lymphocytes recently stimulated by antigen. The relative contribution of each of these two general processes can be easily determined for any serum protein if the catabolic rate is known, i.e. the rate at which the particular protein is being eliminated from serum assuming that equilibrium between the plasma and extra-vascular fluids has been already established. The half-life of IgE in the mouse was not known at the time this study was initiated; the main obstacle being the extremely low concentration of IgE in mouse serum and the lack of availability of a murine IgE-secreting myeloma.

The half-life of plasma IgE was determined by following the decline of biological activity (Figure 3). A mouse antiserum was obtained which showed a high PCA titre for OA; by injecting this serum into normal mice and assaying serum samples taken at regular time intervals thereafter for biological activity, the rate of disappearance was determined.

The disappearance of passively acquired IgE antibody from the circulation of normal animals could have been due to immunoglobulin catabolism and/or to binding of IgE to tissue

mast cells and basophils. Although both mechanisms likely play a role in the clearance of the injected IgE, catabolism appeared to be dominant in the experiments described in these studies. Thus, the elimination of IgE antibody appeared to be unimodal from 1 hour to 96 hours, suggesting that a single mechanism of elimination was operative. Tada et al (34) previously showed, in the rat, that tissue-binding was complete after 24 hours or less. Moreover, these workers using a similar approach have reported that the half-life due to catabolism of rat IgE was independent over a wide range of concentration of rat IgE injected. It is worth noting that the half-life of 10.5 hours is quite similar to the figure of 12.5 hours reported by Tada for rat IgE. Recently, some four years after this thesis work was completed two studies have been made on the catabolic rate of mouse IgE in mice using monoclonal antibodies. A value of 12 hours was reported in both instances. The data derived by Hirano et al (149) was obtained by following the loss of biological activity from serum whereas that obtained by Zheng et al (150) was based on the loss of radioactivity from the serum of mice injected with 125-I-labelled monoclonal IgE.

In the face of this rapid catabolic rate of mouse IgE, maintenance of the virtually steady state of IgE anti-OA levels observed over a period of several months must involve a significant and near-constant addition of newly synthesized IgE from some cellular source. In the initial phase of these studies the most likely source considered for this newly

synthesized IgE was a pool of recently produced plasma cells. It was assumed that since the antigen used for immunization had been administered in $Al(OH)_3$ there would be significant persistence of antigen and this would be responsible for the assumed protracted recruitment of cells to IgE production. Although at the time there was reluctance to accept the concept of a population of long-lived AFC it offered a closer fit to the actual data. Even if antigen had persisted from the time of immunization it would be necessary to make a further assumption that the concentration was not declining significantly during a period of 8 to 12 months.

## 4.2 Antigen-independent adoptive transfer of anti-OA production

A conspicuous feature of the BDF1 anti-OA response is the extended period over which the antibody level remains at a near constant level (Figure 1). Although not unique in this regard, KLH also gave a protracted primary response and several antigens elicited sustained secondary responses, OA was clearly the most potent of the several antigens tried (Figure 2). In view of the above observations, an attempt was made to establish an adoptive transfer system that would facilitate an examination of the cellular events underlying the sustained production of IgE anti-OA antibody. Exploratory experiments involving injection of cells from BDF1 mice previously immunized with OA, into irradiated recipients led to the discovery that injection of OA into the recipients

was not a prerequisite for the initiation of IgE anti-OA production in these mice (Figure 4). The findings from this system which warrant discussion are the kinetics of the response and its duration. In the irradiated recipients there is evidence of some antibody production immediately following cell transfer, however this quickly declines to below the level of detectability suggesting that any antibody forming cells transferred either die or simply stop secreting antibody (Figure 27, day 3). The antibody level subsequently rises steeply from 5 - 7 days after cell transfer to reach a level which remains constant for many months. The rapid rise in PCA titre strongly suggests that cell proliferation was the basic mechanism underlying antibody production. An alternative possibility would be that the AFC transferred to irradiated recipients underwent, in the host animal, a transient cessation of antibody secretion and that on recovery of this property the IgE anti-OA levels rose again to some constant level. The first point to be addressed experimentally however was to establish whether or not the anti-OA measured in the recipients was indeed of donor origin.

Evidence which suggested that this was the case came from a comparison of antibody levels in donor animals with those achieved in recipients. A positive correlation was shown between PCA levels in the donors with those in the recipients (Figure 14). Also, the anti-OA titres in the recipient animals were roughly proportional to the number of spleen cells transferred (Figure 11). Additional evidence,

which will be discussed also later in a different context, stems from the radiation sensitivity of the donor cell preparation. For example when donor lymphocytes were subjected to 600 rads of gamma-irradiation in vitro immediately prior to injection into the irradiated recipients no antibody was detected in the recipients (Table 22). This finding clearly pointed to the donor cells, or their progeny, being the source of the IgE antibody found in the recipients. In the event that the donor cells carried OA into the recipients, it could be argued that this antigen perhaps stimulated endogenous antibody production in the recipients. This argument was minimized when it was observed that irradiated mice were unable to respond to immunizing doses of antigen given from 1 to 14 days after irradiation (Figure 10).

Mice immunized with OA produced IgE antibodies over an extended period and cells transferred from these animals to irradiated recipients produced IgE antibodies also over a prolonged period. OA was the most potent antigen in BDF1 mice in that it elicited the highest PCA titres following primary immunization. However, secondary antibody responses to amylase, KLH and DNP all gave high PCA titres and were sustained over a period of several months. Despite the persistent responses elicited by these three antigens, antibody was not detected in irradiated recipients following transfer, of spleen cells from appropriately immunized donors (Figures 7,8 and 9). This raised several questions regarding the basis for the transferred anti-OA response. The

experience gained from a number of different experiments suggested that (a) FCS constituted an inconsistent requirement for the production of the adoptive response (b) there was no significant cross-reaction, at the antibody level, between OA and FCS and (c) although antigens other than OA failed to produce an adoptive response they did lead to sustained antibody responses in antigen-immunized mice. The role of FCS in the adoptive response will be taken up again later in this Discussion. Since in some adoptive transfer experiments high anti-OA titres were produced in recipients in the absence of FCS the possibility was considered that mice were receiving OA by some other route, perhaps by way of chicken products in their food. Several attempts were made to demonstrate some reactivity between anti-OA and saline extracts of the pelleted mouse chow; none could be shown. The manufacturers of the mouse chow provided an analysis of their product and stated that no chicken products were used in its manufacture. This may not be a relevant issue and the variability with respect to the apparent FCS dependence may, in fact, be due to different quantities of OA being associated with the cell suspensions prepared from donor mice.

In a series of studies Tew and colleagues (151-153) demonstrated that antigen could remain associated with lymph node cell suspensions for several months after immunization. The authors suggested that the cell which played a crucial role in retaining antigen in an immunogenic form was the follicular dendritic cell. In relation to the specific

question of IgE anti-OA production Holt et al (136) have confirmed the findings reported in this thesis, which have been published also elsewhere (154,155), and have shown using BALB/c mice that a nominally antigen independent anti-OA response was produced in irradiated recipients of spleen cells from OA-immunized donor mice. An aspect of this system which might reveal something of its underlying mechanism was the rate at which donor cells, capable of transferring an antigen independent response, were produced in the donor animals. It could be envisaged that antigenic stimulation of donor animals led to the production of a population of proliferating cells which simply completed their differentiation in the recipients to give rise to a population of resident AFC. If this were the case some correlation would be expected between the kinetics of the donor antibody response and the magnitude of recipient responses. In other words antigen-stimulated cells would manifest their activity in both donors and recipients. When this aspect of the system was examined systematically it became evident that for the first four weeks after injection there was no defined relationship between donor antibody levels and the capacity of donor cells to produce IgE antibody in the irradiated recipients (Table 2). As described in the Results section the PCA titres in donor animals reached a maximum value 10 days after immunization and then subsided to a steady state level equal to approximately 50% of the maximum value. At the time when the antibody level in donor mice was at a maximum the spleen cells from these mice showed

essentially no capacity to develop an adoptive response. PCA titres in recipients of 10 day cells (post-immunization) were less than 10% of the value produced by cells taken several weeks after donor immunization. It may be concluded from this asynchronous production of IgE antibody secreting cells and of precursors of the cells which give rise to the long term response in irradiated recipients that they either have different precursors or develop from a common precursor with marked asymmetry with respect to the relative proliferation and/or differentiation rates. The development of the protracted phase of the response in immunized mice shows some degree of coincidence with the development of cells with the capacity to support the protracted response in irradiated animals. This may be merely coincident development, or it could be that the progenitors of the protracted response in non-irradiated mice and those of the protracted response in irradiated mice are one and the same population. The kinetics of development of these cells are not unlike those of B memory cell development as described by Cunningham and Sercarz (156); this study suggested that 6 weeks were required following immunization for full development of the B memory population. A more extensive discussion of the role of B and T cells in the adoptive response will be presented later.

To determine whether the host animal could play an active role in the adoptive response, for example by providing cells which would be recruited to antibody production and perhaps augment the response, normal BDF1 mice were used as

adoptive recipients. When spleen cells taken 6 weeks after immunization of the cell donors, which were actively producing IgE anti-OA, were transferred to unirradiated recipients no evidence of renewed IgE anti-OA production at 5 - 7 days, or later, after transfer was observed. In effect, the adoptive response was completely suppressed. To determine whether this was an active response on the part of the recipient animals an attempt was made to inactivate the suppressive mechanism by subjecting the recipients to graded doses of whole-body irradiation (Figure 16). Results of these experiments showed that although some IgE antibody production did occur at low doses of irradiation significant responses were seen only at 450 rads and above. Although these data were consistent with an active suppressive mechanism direct attempts to demonstrate this active mechanism with cell mixing experiments were unsuccessful. When normal thymocytes were mixed with an active spleen cell suspension, i.e one prepared six weeks after donor animal immunization, and the two were injected into irradiated mice - no suppression of the adoptive IgE anti-OA response was evident (Table 3). Increasing the ratio of normal thymocytes:active spleen cells from 1:4 to 1:1 still failed to reveal any evidence of suppression. Spleen cells from normal donors were also inactive in this system.
It may be concluded from these observations that the suppressive mechanism in normal mice acting on an adoptive response does involve directly or indirectly cell proliferation, since irradiation clearly abolishes the

suppressive effect. The suppression may not be related to suppressor lymphocyte function since neither spleen nor thymus seem to offer a source of suppressor cells; possibly the number of lymphocytes used was not high enough to be effective

There is no doubt however that the anti-OA response transferable to irradiated recipients is suppressible by T cells. Holt et al (157) have shown, that T cells from the spleens of OA-tolerant BALB/c mice, when injected together with spleen cells from OA-immunized mice into irradiated recipients, will suppress the development of this nominally antigen-independent adoptive response. Certain aspects of this finding will be discussed later in relation to the role of B and T cell proliferation in this system.

## 4.3 Initiation of the adoptive response: mitogenic and antigenic properties of FCS.

In the initial series of experiments using the adoptive transfer system FCS was incorporated routinely into the tissue culture medium to ensure high cell viability. FCS is not a standardized reagent in the sense that some batches will support antibody responses in vitro very effectively whereas others appear to be lacking in this property (158). It was not until numerous experiments had been completed over a period of several months that some pattern emerged with respect to the role of FCS in the adoptive transfer system. Two general conclusions emerged: (a) FCS was not an absolute requirement for IgE anti-OA production, in about 30 - 40% of

all experiments in which FCS was not incorporated into the tissue culture medium IgE anti-OA production nevertheless was observed in the irradiated recipients, and (b) in those experiments which revealed a requirement for FCS it was possible to identify "supportive" and "non-supportive" batches of sera.

The action of FCS was considered to be due either to antigenic cross reactivity with OA or to the mitogenic capacity of one of its constituents. Antigenic similarity between OA and a component of FCS was considered unlikely because not all batches of FCS were supportive of the adoptive response (Table 4). An attempt was made nevertheless to demonstrate similarity between OA and supportive FCS. Within the limits of sensitivity of the inhibition of PCA assays employed for this purpose, no evidence of antigenic similarity was observed (Table 6). However, the possible presence of OA-like material in FCS was only one of the two basic parameters of the adoptive response, the other was the sensitivity of the response to OA itself. In other words, what was the minimum quantity of OA required to initiate an adoptive response? This particular question was addressed by transferring fixed numbers of cells from appropriately immunized donor along with graded doses of OA (Figure 20). From the data obtained it was evident that as little as 1 ng of OA could elicit an IgE response equal in magnitude to that of medium containing supportive 20% FCS. It should be noted that the assays used in the previously described attempts to

demonstrate antigenic similarity between OA and FCS were not sensitive to OA at this low level. It must be concluded therefore that, on the basis of the experiments described, antigenic similarity between OA and FCS cannot be excluded as an explanation of the role of supportive batches of FCS in facilitating a transferred response.

This avenue of investigation suggested that antigenic components which are intrinsic constituents of FCS are not related antigenically to OA since not all FCS batches were supportive. It allowed nevertheless that a mitogenic activity could be associated with certain batches of FCS and not others. An attempt to demonstrate the presence of a mitogenic factor in FCS was made by exposing mouse spleen cells to different concentrations of both supportive and non-supportive batches of FCS in vitro and determining the extent of proliferation (Table 9). It was evident from the data obtained that the capacity of a given batch of FCS to support an adoptive anti-OA response did not correlate with its mitogenic activity. The capacity of different batches of FCS to induce cellular proliferation, as measured by 3-H thymidine uptake was an independent function of their capacity to support the adoptive response.

A different tack was taken by posing the question of whether compounds of known mitogenic activity could facilitate an adoptive anti-OA response. Spleen cell suspensions were mixed in vitro with mitogen over a range of concentrations and the entire suspension injected into irradiated recipients

(Figure 21). Under these conditions, the B cell mitogens LPS and dextran sulphate both elicited adoptive responses, the optimal concentration of each produced responses of approximately 10 - 25% of that seen in the FCS control animals. The T cell mitogens Con A and PHA did not, over the entire concentration range tested, elicit any detectable IgE antibody. PWM, which has been reported to affect both B and T cells elicited a low IgE response, of the order of 5% of the FCS control. In summary, it seems from these data that recognized T cell mitogens are not effective whereas recognized B cell mitogens are marginally effective in eliciting adoptive IgE responses in the model system under study. From the data obtained in these two series of attempts to identify antigenic and/or mitogenic activity, i.e. supportive factor, in FCS it was concluded that (a) a mitogenic factor was not responsible for the supportive properties and (b) antigenic similarity between OA and FCS could not be ruled out since the sensitivity of the test used to demonstrate this was too low. If we accept the conclusion that OA and FCS did contain a common antigenic component then, as mentioned previously, that component cannot be an intrinsic constituent of FCS. Justification for making this statement is based on the observation that not all batches of FCS were supportive. Furthermore, attempts to fractionate by gel-filtration and thereby isolate an active component from FCS led to the finding that supportive activity was relatively evenly distributed over a molecular size range from 70,000

daltons to about 350,000 daltons (Figure 24, Table 11). This observation suggests that supportive FCS contains perhaps several active components (132) and since the activity is associated with solutes of molecular weight greater than that of OA (44,000 daltons) it appears improbable that OA or a component similar to OA is one of these.

In view of the above findings it is proposed that the variability which was evident between experiments where the known variables were (a) the batch of FCS and (b) presence of FCS, was due not to the properties of the FCS itself but to variations in the amount of antigen carried over in a cell-bound form.

## 4.4 Persisting antigen and the effect of antibody on the adoptive response

Other model systems have been described in which "spontaneous" antibody responses can be demonstrated upon adoptive transfer of lymphoid cells into unimmunized mice (133-136,159). In the original report (133) Mitchison transferred spleen cells from antigen-primed mice into irradiated recipients. After an initial induction period the transplanted cells began spontaneously to produce antibody to the immunizing antigen without further overt antigenic stimulation. However, spontaneous responses occurred only if an in vitro incubation period at 37°C preceded the adoptive transfer. It was suggested that in vitro incubation was required for unmasking of persistent antigen. However, the

unmasking of cell associated persisting antigen should occur in the adoptive host as well as in vitro. In the studies described in this thesis it was shown that the in vitro incubation step was indeed unnecessary (Figure 5). This finding has been subsequently confirmed in two other systems (154,160). In the report of Phipps et al (160) substantial spontaneous responses were consistently induced when draining lymph nodes from immunized mice were teased into single cell suspensions and transferred directly into irradiated recipients. In all studies of "spontaneous" induction, good responses could be generated even though the donor mice had been immunized months prior to the adoptive transfer. The induction of a spontaneous response was attributed to antigen which persisted in immunogenic form on the surface of an accessory cell. It was postulated that when lymphoid cells were removed from the antibody-rich environment in vivo and placed in an antibody depleted environment in vitro, persisting antigen associated with accessory cells could stimulate primed cells to produce detectable antibody specific to the immunizing antigen (135). Lymphoid organ fragments from immunized animals have also demonstrated spontaneous antibody responses (161,162). When lymph nodes, from animals immunized months previously with antigen, were cut into small fragments and cultured in vitro, spontaneous antibody responses were induced. As with the adoptive transfer and cell suspension systems referred to previously, spontaneous responses in organ fragment culture were attributed to

stimulation of memory-B cells in fragments containing cell-associated persisting antigen.

It would be predicted that if indeed, in the adoptive IgE anti-OA response of BDF1 mice described in this thesis, antigen were crucial to the initiation of the response then injection of specific antibody into the adoptive recipient should inhibit the response. This prediction was borne out. Injecting anti-OA antibody into irradiated recipients shortly before cell transfer effectively suppressed the transferred response. An intriguing aspect of this finding was that serum antibody from the prospective cell donor, taken immediately before the mice were killed to provide spleen cells, also effectively suppressed the adoptive response in recipients (Figure 27). There existed then an apparently paradoxical circumstance wherein the donor mice were actively producing high IgE anti-OA titres and IgG concurrently, yet when a small volume of this serum antibody was injected into irradiated recipients before transfer of cells from the same animal, i.e. the serum antibody donor, the adoptive response was suppressed. Two conclusions were drawn from this result (1) antigen was implicated in the initiation of the secondary adoptive response and (2) the cellular basis for antibody production in the cell donor, at the time of cell transfer, had a different basis from that existing in the recipient, at the time the response was being initiated. Suppression of the adoptive response was brought about by a concentration of antibody in the adoptive recipient which was

no more than 20% of the level in the donor animals, in which IgE antibody production was continuing without any obvious decline in titre. This result suggested that antigen was indeed required for the initiation of the adoptive response but perhaps not obligatory for the long term sustained phase of the IgE anti-OA response in the prospective cell donors. Several attempts were made to identify the stage at which antibody was acting. In vitro pulsing of cells with antibody followed by transfer of the washed cells into adoptive recipients showed that antibody was effective as an inhibitor at this stage, but not in so striking a fashion as with in vivo administration (Figure 28). These data were thus consistent with antigen being associated with one or more cell types implicated in the adoptive response. As referred to previously, other reports indicate that antigens do persist for extended periods, for example bovine immunoglobulin is detectable in the liver of mice more than 3 months after immunization (163) and Tew and Mandel (162) have reported that antigen localized in lymphoid follicles can persist for long periods. Also, evidence was described which implicated this antigen in the antibody response given in an adoptive transfer system.

Although the role of the Fc region of the suppressive antibody was not examined in detail, it is noteworthy that a role for this region of the molecule has been described for both antigen initiated and mitogen induced responses. Sinclair demonstrated that the Fc region of

specific antibody played an important role in determining the extent of suppression, $F(ab')_2$ was much less efficient as an inhibitor of an antigen initiated antibody response than was intact antibody (164). Similarly, Ryan et al (165,166) showed that mitogen (LPS)-induced proliferation of lymphocytes could be inhibited by surface immobilized antibody. In these reports inhibition of the response was not evident if $F(ab')_2$ fragments were used, thus implicating the Fc fragment and, most likely, the Fc receptor on B cells. It may be stated parenthetically that reports have been published which indicate that LPS can affect T cell function (167,168) and that a sub-population of T cells also may express Fc receptors (169,170).

## 4.5 Cellular components of the adoptive anti-OA response.

When the first report of the work described in this thesis was published (154) it was considered most probable that the basis for the sustained IgE anti-OA response in BDF1 mice was continued differentiation of lymphocytes to antibody secreting cells by persisting antigen. However, since a limited number of reports had suggested that antibody producing cells could (a) persist for several months (171) and (b) arise under some circumstances without the usual extensive proliferative phase (144,145), it was considered essential to study this aspect of the system in some detail in order to clarify the cellular basis for both the sustained response in intact animals and the adoptive response in irradiated

recipients.

The previously discussed experiments using specific antibody had suggested that antigen was associated with the cells transferred into irradiated recipients. The initial efforts to unravel the cellular interactions involved in the adoptive response were therefore directed towards identifying a cell type which was considered likely to be antigen-bearing. The role of macrophages in this regard was investigated by depleting spleen cells from appropriately immunized donor mice of macrophages and determining the capacity of the remaining B and T cell populations to mount an adoptive response. Since specific methods, i.e. methods which isolated exclusively macrophages, were not readily available, non-specific methods were employed. Spleen cell suspensions prepared from mice 6 - 10 weeks after immunization were allowed to adhere to Petri dishes, were passed through columns of Sephadex G-10 or were centrifuged through a discontinuous density gradient. These methods were effective in removing macrophages. The data were quite clear in that macrophage-depleted spleen cell suspensions produced anti-OA titres in irradiated recipients which were not measurably different from the unfractionated starting cell suspension (Table 15). If macrophages did express membrane bound antigen it was evident from the results that it was not essential for the initiation of the adoptive response.

Further efforts were devoted to determining whether T cells played a central role in this system. T cells were

removed from spleen cell suspensions, by treatment with anti-Thy.1 and complement; the remaining viable cells were isolated and injected into irradiated recipients (Figure 31).

Cells treated with normal serum and complement produced an anti-OA PCA titre of 2048 by 8 days after treatment, which had not changed significantly by day 17 (PCA titre = 1024). Cells treated with anti-Thy.1 and complement failed to produce any detectable anti-OA for at least 17 days; the adoptive response was evidently strongly T cell dependent. T cells were not simply antigen bearing cells since mitomycin C treatment of isolated T cells also prevented antibody production in the recipients (Table 20). Mitomycin C may have exerted its inhibitory effect by more than one mechanism of action. By inhibiting DNA replication it may have prevented necessary cellular proliferation. Alternatively, by interfering with the production of mRNA, mitomycin C may have prevented the production of an essential T cell factor which would subsequently act on B cells to induce antibody production. Exposure of primed B cells to mitomycin C also inhibited the transferred response. To assess the requirements for proliferation a dose-response experiment was then carried out to determine the radiosensitivity of the cells underlying the adoptive response. The in vitro radiation sensitivity exhibited by the transferred cells was generally consistent with previously published figures on proliferating lymphocytes in which D37 values of the order of 100 rads were observed (172,173). Lymphoid cells exposed in vitro to ionizing radiation can manifest subtle changes in their

migratory properties following adoptive transfer to irradiated hosts (174). On the other hand mitomycin C treatment of lymphoid cells has been reported not to produce a significant alteration of migratory properties in a similar adoptive transfer model (175). The data reported in this thesis have been interpreted to mean that there is a need for activation and proliferation of B and T cells at least to initiate antibody production in recipients. The nature and locus of the stimulus for activation remains unclear.

Although proliferation of both B and T cells is evidently an important feature of the adoptive response during its inductive phase, the attainment of a stable level of anti-OA is accompanied by marked radioresistance of antibody production. Groups of animals which had been actively immunized with antigen and adjuvant and also mice which had been adoptively immunized were both exposed to whole-body irradiation ranging from a dose of 200 rads to 1,000 rads (Table 21). The marked radioresistance of an established anti-OA response was evident in both groups of mice. Since the radiation doses administered were relatively high, it is likely that a major fraction of those cells which would normally proliferate in response to antigen would be prevented from completing mitosis (176,177).

A similar pattern of long-term radioresistant antibody formation has been reported by Okudaira (178) and recently, using the experimental system developed in the present study, also by Holt et al (157). Direct evidence for

the continued production of anti-DNP antibody by a secreting cell population was described by Okudaira and Ishizaka (178), who showed that T cell depleted spleen cells withstood 1,000 rads and continued to secrete antibody in vitro following removal from mice 8 weeks after immunization.

The adoptive transfer system displays a pattern of sensitivity followed by marked resistance not only to the effects of irradiation but also to the effects of suppressor T (Ts) cells. Holt and Leivers (157,179) have recently reported that the inductive phase of the adoptive response is susceptible to suppression using T cells from OA-tolerant mice. When the Ts cells were transferred to irradiated recipients at the same time as an equal number of OA-immune cells the adoptive response was suppressed by about 90%. On the other hand when injection of the Ts cells was delayed by 30 days after injection of the OA-immune cells there was no ensuing change in the PCA anti-OA titres suggesting that the effector phase of the response was quite resistant to the effects of Ts cells.

One of the more interesting studies of the last few years on IgE regulation in mice was the work of Takatsu et al (160). The authors described how repeated injections of urea-denatured OA into OA-primed mice induced antigen specific suppressor T cells which suppressed both the primary and secondary IgE antibody responses to native antigen (180). Once a persistent antibody response was established , however, the same treatment was not so effective in depressing the

level of IgE antibody. A reduced or weak effect of suppressor T cells on an established response suggested that some mechanisms other than recruitment of B memory cells was involved in the maintenance of the antibody level. In the more recent work of Okudaira and Ishizaka it was shown that depletion of B memory cells by exposure of antigen-primed mice to a lethal dose of X-irradiation failed to terminate established antibody production of both IgE and IgG isotypes, and suggested, as referred to earlier, that radioresistant long lived antibody forming cells are involved in this process (178). Somewhat similar data were reported by Bach and Brashler who showed that aceto-acetylated-OA, as a second example of a modified antigen, also would prevent a secondary IgE antibody response induced by native antigen (181). It was shown also in this latter report that the suppressive effects of the modified antigen persisted for up to 2 months.

The data cited above thus support the idea of there being two phases to an IgE response elicited under appropriate conditions. First would be the antigen induced acute proliferative phase, which is suppressible by Ts cells elicited by high doses of chemically modified antigen. Second would be the protracted phase which, would display marked resistance to irradiation and suppression by Ts cells elicited by either native or chemically modified antigen. It is this latter phase which Okudaira and Ishizaka have attributed to a long lived plasma cell population (178).

The demonstration of long lived plasma cells was

previously reported by Miller (171), who estimated that the life span could be considerably longer than 6 months. This author concluded, on the basis of autoradiographic studies, that the bulk of antibody, produced at extended timed after immunization was being made in cells developed shortly after the original antigenic stimulus. The production of a population of long lived antibody secreting cells is evidently related to the dose of antigen used for immunization. High doses of antigen lead to only transient responses (182), whereas low doses of antigen elicit sustained responses (183).

Conceivably, proliferation of the lymphoid cell precursors of the long lived antbody secretors is suppressed by high antigen dose immunization, since suppressor T cells are known to be formed under such conditions (183,184).

The entire question of continuing regulation becomes a problematic one with regard to a pool of long lived antibody secreting cells. Suppression of the response at an early stage may be occurring with high antigen dose immunization. However, since a salient feature of the long term response to immunization with low dose of antigen is the virtually constant level of detectable antibody, the mechanism by which this level is regulated are not so obvious. Of possible significance in this context is a report by Schrader (185) which suggested a direct role for antigen in regulating the secretion of antibody by plasma cells. The difficulty with this mechanism is that for the production of long term responses typically extremely low doses of antigen are used

for immunization. Indeed, in allergic humans it has been estimated that a total seasonal dose for a typical pollen allergen is no more than 1 μg (148). A germane question in this context is whether, in humans, there are any examples of long lived plasma cells.

## 4.6 Persistent, radioresistant IgE production: is there a parallel in humans?

At the present time there is considerable debate as to whether the in vitro systems currently being used for the study of IgE synthesis by human cells are indeed adequate for demonstrating de novo antigen-induced activation (186). Notwithstanding this reservation Saxon and Stevens (187) have reported that peripheral blood leukocytes from atopic patients contain a population of B cells which produced IgE in vitro without further antigenic stimulation. More than 75% of the IgE synthesized was produced in the first 72 hours and did not occur if cycloheximide was included in the culture. The spontaneously secreting cells (SCC) were found in all six patients tested but were not invariably present, the SCC were not detected in three out of fifteen experiments. It is noteworthy that patients' T cells did not inhibit IgE production in 7 out of 13 experiments. Other studies from the same group (188) have shown that IgG anti-tetanus toxoid also may be synthesized in vitro by peripheral blood B cells in the absence of T cells or mitogen. However, in this last report the particular B cell sub-population which produced the

T cell independent IgG appeared only transiently and persisted for about 10 days only following booster immunization of the normal volunteers.

In the case of asthmatics, cultures of peripheral blood leucocytes depleted of E-rosetting T cells developed IgE antibody to mite allergens in the absence of added antigen or pokeweed mitogen. Significantly, in relation to the data presented in this thesis, it was observed that in vitro high dose irradiation (1,000 rads) had no effect on the production of IgE antibody in 6 out of 8 cases. In the remaining 2 cases the level of IgE was reduced by 50% (189). These conclusions were confirmed in a further study in which it was reported that a highly differentiated set of IgE secreting B cells existed in the peripheral blood leukocytes of a group of patients with atopic asthma; these B cells were able to produce IgE protein and specific anti-mite antibody in vitro in the absence of antigen and without T cell help (190). It was further reported in this last study that this sub-population of B cells can be specifically tolerized with antigen without active suppressive mechanisms.

The studies described in this thesis thus appear to be significant with reference to the reports on IgE production in vitro by human cells. However, as alluded to earlier, there are differences of opinion with respect to what constitutes IgE synthesis in vitro in contrast to a simple release of IgE in tightly bound form from peripheral blood leukocytes. Recent data suggests that some methods are

beginning to emerge which will allow resolution of this particular problem (191).

Finally, it should be emphasized that in the studies described in this thesis using mice and from the data referred to on IgE production by human cells it is clear that distinct sub-populations of B cells must exist. At least two major populations can be identified, one subserving the acute phase of the IgE response by producing short-lived plasma cells and a second subserving the chronic phase of the response by producing, also through a process of proliferation, a set of long-lived cells. It is not known whether these sup-populations arise as a consequence of sequential or parallel lines of development from a common precursor. The sequential model fits with the observations made on the kinetics of development of the IgE antibody response itself and also of cells capable of producing an adoptive response in irradiated recipients. However, the separate-lines model is not excluded by these data.

There is a paucity of information available on murine B cell ontogeny in general that would facilitate an understanding of this problem. It is known that isotype expression on the cell membrane changes during B cell development from (high IgM:low IgD) to (low IgM:high IgD) (192). Also, there are functional changes such as a decrease in susceptibility to tolerance induction (193) and changes in the expression of differentiation antigens (194). It is known, for example, that the membrane antigen Lyb.5 is

expressed on B cells from normal mice. But, male CBA/N mice which express a mutant allele at the xid locus do not express the Lyb.5 antigen on their B cells and this defect coincides with an inability to respond to type 2 (thymus independent, type 2) antigens such as TNP-Ficoll and SSIII, a type-specific pneumococcal polysaccharide antigen (197). It is not known whether the Lyb.5(-) cells in CBA/N xid mice are identical to the Lyb.5(-) cells found in normal mice. As was discussed above in relation to the development of IgE responses and the ability to develop an adoptive response, so with the relationship between Lyb.5(-) and Lyb.5(+) B cells it is not known whether the cells have a common precursor or whether they develop in sequential fashion, i.e. Lyb.5(-) differentiates to an Lyb.5(+) stage. In any event this data on B cell sub-populations does not offer much help in understanding the B cell heterogeneity evident with respect to the IgE response to OA since B cells which are Lyb.5(-) do not respond to thymus dependent antigens, such as OA. It is evident that a more detailed analysis of the B cell sub-populations subserving the IgE anti-OA response in BDF1 mice must await further investigation of B cell heterogeneity.

It is possible to postulate that both sets of B cells are produced to provide maximal immune protection to an organism. Following exposure to a potential pathogen the rapid production of a high concentration of antibody could serve to terminate rapidly the infection or infestation. Although the production of memory B and T lymphocytes would

ensure a more prompt response and production of antibody should a further exposure occur to the same pathogen, it could be argued that the presence of antibody at that time would serve more efficiently to prevent reinfection or infestation. In the case of IgE, given that the half-life of circulating antibody is very short and that cell bound IgE has a half-life less than that of plasma IgG, it would be advantageous for the animal to maintain circulating IgE levels over a long period of time by having antibody synthesized and secreted by long lived plasma cells. It can be argued that IgE antibody in plasma probably plays a negligible direct protective role because of the great difference in concentration between IgE and other immunoglobulin classes, the IgE plasma concentration is at most 1/1000 that of plasma IgG. The major action for IgE antibody is expressed through a biological amplification system involving mast cells, basophils and the several vasoactive substances which these cells secrete following interaction of cell bound IgE with antigen. Despite the fact that cell bound IgE has a half-life approximately 20 times greater than that of plasma IgE the two forms are in equilibrium (48) and continued synthesis is required to maintain the cell bound form at an effective concentration.

In summary, it is possible to offer what appears to be a rational speculation for the existence of a long-lived antibody secreting cell. The validity of these speculations must, of course, await further data on the functional role of IgE.

# Appendix A: List of Figures

# Appendix B: List of Tables

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