THE UNIVERSITY OF MANITOBA

ANTIBODY PRODUCING CELLS IN TRANSPLANTATION IMMUNITY

by

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF IMMUNOLOGY

WINNIPEG, MANITOBA

AUGUST, 1973

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. E. Sabbadini for his unfailing guidance and encouragement throughout the course of this study. Special thanks are due to Dr. S. Fujimoto for his assistance in the preparation of the rabbit anti-mouse gamma G antiserum. I wish to thank Dr. H. Tsay for the gift of the anti-Kappa type Bence Jones protein antiserum. I am grateful to Dr. G. Goldenberg of the Manitoba Cancer Treatment and Research Foundation for the gift of the α-methyl-D-mannoside. Finally, I wish to thank Miss Amy S.P. Chan for her extreme patience in typing this thesis.

ABSTRACT

Jerne's hemolytic plaque method was modified and applied to the study of antibody producing cells in transplantation immunity. Using allogeneic red blood cells as indicator cells and fresh normal rabbit serum as the complement source, a peak response (on day 6 after secondary immunization) of some 2000 PFC per spleen was obtained across a strong histocompatibility barrier. The use of rabbit complement was considered to be the major factor in the relatively high sensitivity of the method. Experiments were performed to investigate the antibody classes involved in the donor-recipient combination under study. Immunologic specificity of plaque formation was thoroughly examined and the method was found to be highly specific.

ABBREVIATIONS

A	A/J strain
AKR	AKR/J strain
$B6AF_1$	$(C57BL/6J \times A/J)F_1$
B10	C57BL/10J (or B10/J) strain
B10.A	B10.A/J strain
B10.BR	B10.BR/J strain
B10.D2	B10.D2/J strain
C	Complement
CBA	CBA/J strain
CFA	Complete Freund's adjuvant
C57BL/6	C57BL/6J strain
CMI	Cell-mediated immunity
Con A	Concanavalin A
C3H	C3H/HeJ strain
DA	Developing antiserum (= rabbit anti-mouse gamma G antiserum)
DBA/1	DBA/1J strain
DBA/2	DBA/2J strain
FCS	Fetal calf serum
GPS	Guinea pig serum
HAP	Humoral antibody production
i.p.	Intraperitoneally
KBJP	Kappa type Bence Jones protein
$M\gamma G_2$	Mouse gamma G_2 immunoglobulins
MγM	Mouse gamma M immunoglobulins
MRBC	Mouse red blood cells
MWS	Mouse whole serum
O.D.	Optical density
PFC	Plaque forming cells

PBS	Phosphate buffered saline
RAMRBC	Rabbit anti-MRBC antiserum
RBC	Red blood cells
RC	Rabbit complement
RFC	Rosette forming cells
RS	Rabbit serum
Sal	Sarcoma 1
SRBC	Sheep red blood cells
VCN	*Vibrio cholerae* neuraminidase

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INTRODUCTION

Histocompatibility can be looked upon as a measure of genetic disparity between individuals in relation to the conditions determining the survival or the rejection of a graft exchanged between them. Transplantation or histocompatibility antigens are the products of histocompatibility genes and are present on the cell surface. Cells or tissues, after grafting to a foreign host, induce an immune response in the host, resulting in the rejection phenomenon. This immune response, therefore, is a function of the genetic difference between donor and recipient, which entails a greater or lesser degree of histoincompatibility.

Histocompatibility antigens may induce the stimulation of at least two types of immunocompetent cells, i.e., those responsible for cell-mediated immunity and those responsible for the secretion of humoral antibody. The available data point to the fact that neither of them is exclusively represented in an immune response, although their relative significance in any particular case may vary. For example, cell-mediated immunity is of paramount importance in skin graft rejection, but yet, the possible participation of humoral antibodies in the rejection phenomenon cannot be excluded.

LITERATURE REVIEW

THE H-2 SYSTEM IN MICE

It has been established that for a particular species there is at least one strong histocompatibility locus, the gene-products of which are of particular importance in histocompatibility. Examples are: the Ag-B (H-1) locus of rats (Elkins and Palm, 1966), the B locus of chickens (Crittenden et al., 1964), the HL-A locus of man (Dausset et al., 1965) and the H-2 locus of mice (Snell and Stimpfling, 1966). A graft which is incompatible with respect to the antigens of the strong histocompatibility locus of the species will be rejected in a relatively short time and the rejection process will be difficult to control with the usual immunosuppressive therapies. In addition to such strong histocompatibility loci, many weak histocompatibility loci are known to exist for each species. A difference at one or several weak histocompatibility loci between the donor and the recipient of a graft will also result in rejection; however, the rejection times involved may be longer than those for strong histocompatibility differences.

In the study of transplantation immunity, the mouse has been the animal of choice. The availability of a large number of inbred lines and transplantable tumors and the convenient size and facility of handling of these animals are among the chief reasons for the preference.

Discovery and Early Development of the H-2 System: The H-2 region resides in the 9th linkage group (Gorer et al., 1948) in mice. Gorer showed that Antigen II, a cellular antigen discovered with the aid of a rabbit anti-mouse antiserum, was of prime importance in tumor grafting and blood typing (Gorer, 1937, 1938, 1942). This Antigen II was that of Strong's A strain and, thus, Gorer identified the genotype of this strain as H-2/H-2. Later in 1951, Snell used the method of tumor transplantation and discovered that an animal with the heterozygous genotype $H\text{-}2^k/H\text{-}2^d$ would allow the growth of the A strain tumor 15091a (H-2/H-2) (Snell, 1951). Actually genotypes H-2/H-2, H-2/- and $H\text{-}2^d/H\text{-}2^k$ reacted identically to the tumor. In the case of H-2/- growth of an H-2/H-2 tumor would be expected according to Little's third law of transplantation which states: "Grafts from either inbred parent strain to the F_1 hybrid succeed, but grafts in the reverse direction fail." (See Snell & Stimpfling, 1966). Therefore, the

finding that H-2^d/H-2^k also allowed the growth of such a tumor was interpreted as evidence that H-2^d and H-2^k can complement each other to behave identically as the A strain genotype (now known as H-2^a). Conversely, H-2^a would possess a d and a k component. This was the beginning of the study of the H-2 genetic organization. This biparity was further supported by evidence provided by Allen (1955) and Shreffler (1965).

Serological Studies: The H-2 system is characterized by its readiness to elicit allo-antibody formation, which is difficult to demonstrate for non-H-2 loci (Snell and Stimpfling, 1966). Serological studies, therefore, have played an important part in the understanding of the H-2 system.

The most useful serological technique for studying the H-2 has been haemagglutination, originally developed by Gorer and Mikulska (1954) and later modified by Stimpfling (1961) and Mitchell et al. (1969). The haemagglutination test has the advantages of being technically simple, specific and sensitive, but requires subjective interpretation of the results.

Another test for studying H-2 is the cytotoxicity test of Gorer and O'Gorman (1956). Cytotoxic antibodies against H-2 antigens

are capable of killing specific or cross-reacting target cells in the presence of complement. The number of stained (by supravital dyes, e.g., trypan blue) dead cells is the index of cytotoxicity. A less subjective version of the test was developed by Sanderson (1964) and Wigzell (1965), using ^{51}Cr-labeled cells as targets. Cytolysis is measured by release of radioactive labels into the supernatant caused by specific antibody in the presence of C (usually guinea pig C).

The identification of individual specificities, which have been shown in the H-2 studies to be comprising the different alleles (Gorer, 1938; Gorer et al., 1948; Snell et al., 1953), requires monospecific antisera. For the production of such antisera, absorption procedures, both in vitro and in vivo, have been found to be very useful. For example an antiserum produced by animals of strain X against tissues from strain Y (X anti-Y) may contain antibodies specific for specificities 1, 2, 3 and 4. If a strain Z possesses specificities 1, 2 and 3, but not specificity 4, the absorption of this antiserum with strain Z tissues, either in vitro or in vivo, would selectively eliminate the antibodies against specificities 1, 2, and 3, and the absorbed antiserum would be specific only for specificity 4. In this way, by the judicious choice of donor-recipient combinations and of

the strains to be used for absorption highly specific antisera can be obtained. These antisera can then be used to recognize certain specificities in unknown strains. Since an animal cannot produce antibodies against antigens present in its own tissues, it is evident that the same result (i.e., a monospecific anti-4 antiserum) would be obtained by immunizing the F_1 hybrid generation of strains X and Z with tissues of Y. Thus specificity 4 is defined by the way of producing the antiserum which identifies it. It is obvious that (i) the specificities defined in this way may have no relation with the actual molecular structure of the antigens, and (ii) that an individual specificity may, with further serological analysis, results to be composed by more than one specificity.

By the use of the procedures outlined above, together with some other tests such as leukoagglutination (Mishell et al., 1963) and hemolysis and complement fixation (Winn, 1964), the H-2 system has been shown to consist of 19 alleles and 36 H-2 serologically distinct specificities (Klein and Shreffler, 1971). However, with the study of wild mice (Klein and Shreffler, 1971), it is expected that more antigens and alleles will be detected. The present knowledge of the H-2 system can be summarized in the form of a chart (please see TABLE VII),

which shows all the alleles and their associated antigenic specificities currently recognized. The specificities are usually numbered according to the order of discovery. By the serological analysis of the H-2 antigens in the offspring of hybrid animals, it was shown that intra-H-2 recombination occurs with the frequency of 0.1 to 1% (Klein and Shreffler, 1971). Thus, with the study of such intra-H-2 recombinations, the different H-2 specificities may be arranged in a certain order in the chromosome. It was shown with this type of analysis that the genes controlling the H-2 antigens are broadly arranged into two main regions: the K and the D regions.

The H-2 chart provides a convenient summary of all the known data concerning H-2, but at the same time, fails to present a clear picture as to the real complexity of the immunogenetics of H-2, which will be briefly discussed in the following section.

Genetic Organization: The genetic complexity of H-2 is well known. The recombination phenomenon within the H-2 complex which justifies the individuality of the genetic sites determining different H-2 antigenic specificities was first demonstrated by Amos et al. (1955) and Allen (1955), and confirmed by Pizarro et al. (1961). Furthermore, a few seemingly unrelated traits (to histocompatibility) were discovered

by several authors, the Ss-Slp protein (Shreffler and Passmore, 1971), the "hybrid resistance" phenomenon (Cudkowicz, 1968), the different degrees of immune response to certain tumor viruses (Lilly, 1966), and the degree of immune response to various synthetic antigens, Ir-1 gene (McDevitt and Tyan, 1968). It is very probable that among these, the Ss-Slp and Ir-1 traits map inside the H-2 complex (Shreffler, 1965; Klein and Shreffler, 1971).

The question as to the number of loci involved within the complex has always been a mystery to immunogeneticists: how are these different traits controlled and how are the loci organized if indeed a number of them are involved? The first attempt to solve the problem was the serological analysis undertaken by Gorer and Mikulska (1959). They concluded that as a result of crossing over within the complex, at least four (4) segments in the following order, D, C, V, K, can be recognized. Later in 1961, Pizarro et al. (1961) obtained results which implied that the H-2 complex can be divided into at least two regions, K and D. Stimpfling and Richardson (1965), however, using two new congenic strains B10.A and B10.BR (both congenic with C57BL/10ScSn), showed that at least three distinct regions, D, C, K can be recognized by recombination. The complexity has, up to 1970,

culminated in the studies by Klein and Shreffler, and has shown up to eight subdivisions in the H-2 chromosomal region, viz., D, C, V, E, Ss, A, Ir-1 and K (Shreffler and Klein, 1970). However the same group has brought forth another model depicting the H-2 complex as consisting of only two histocompatibility loci (Klein and Shreffler, 1971, 1972). This new interpretation they called the two-gene model ("hypothesis II"), while the traditional one could be called the multiple-gene model ("hypothesis I"), depicting a system of at least six histocompatibility antigens controlled by regions K, A, E, V, C and D, respectively (Klein and Shreffler, 1972). Using skin grafting in forty-one different strain combinations, they claimed that the experimental results are "unequivocally in agreement with the predictions based on hypothesis II . . ." and ". . .evidence provided by serological, genetic, and biochemical analysis of the H-2 system can be also put forward to support hypothesis II." (Klein and Shreffler, 1972). In a nutshell, it is generally agreed that the K and D regions are the minimal members within the H-2 complex controlling histocompatibility traits. They are physically separated by two seemingly unrelated traits, Ss and Ir-1, with Ss closer to the K end.

At the other extreme, it has been proposed that a single

locus only actually controls all of the antigens (Herberman and Stetson, 1965). Their main argument in favor of this hypothesis is that the expression of H-2 antigens by different subdivisions of the region in various tissues and during development seems to be coordinated. They argued that this can happen only if one single locus only is involved. However, this does not seem to be a unique phenomenon: other antigens controlled by unlinked loci appear to behave likewise; hence, it may be due to regulatory mechanism of higher order, rather than because only one single locus is involved. Moreover, the relatively large recombination frequency within the H-2 region (in the order of 0.5%, Shreffler, 1970; Klein and Shreffler, 1971) points to the fact that more than one locus is involved.

It is obvious that the ultimate solution to the problem of the molecular basis for the genetic control of the H-2 antigens will be obtained only when the products of the histocompatibility genes, i.e., the histocompatibility antigens, themselves could be isolated, purified and characterized. Towards this direction, Nathenson and his collaborators have made a substantial progress. Their work, as reviewed by Nathenson (1970), suggested that there are two glycoprotein fragments carrying either the K or the D end specificities, but not both.

Relations with the HL-A System of Man: The only histocompatibility system of a mammalian species, which has been well studied to the point that it can be compared side by side with the H-2 system is the HL-A system of man. These two systems have been found to be similar in many aspects. As reviewed by Klein and Shreffler, "Beginning with the serological techniques developed for H-2. . . and continuing through discoveries of the role in transplantation, cellular and sub-cellular distribution, development, genetic biparity, intraregional recombination, chemical nature, and presently culminating in speculations about biological function, the HL-A investigations have strikingly paralleled in approach and in results that had previously been shown in studies of H-2." (Klein and Shreffler, 1971). Nevertheless, some striking discrepancies exist. For example, it is generally agreed that the HL-A system appears to be serologically and genetically simpler than the H-2 system. To say that the similarities between the two systems are merely coincidental seems unjustified in view of the overwhelming array of evidence that point to the contrary. Another way to explain the discrepancies concerns the interpretations of the two systems, as discussed in the following section.

The Simple-Complex, Complex-Simple Models: Hirschfeld (1965) suggested it is possible to transform serological reaction patterns into verbal

codes which can be broadly classified into two categories: (i) the complex-simple model which assumes the antibodies to be complex, i.e., heterogeneous and thus cross-reacting with more than one antigenic determinant, and the antigens to be simple, each molecule carrying only a single antigenic determinant; and (ii) the simple-complex model, in which the antibodies are considered to be simple, i.e., reacting specifically with a single determinant, and the antigens to be complex, one molecule carrying more than one antigenic determinant. The author suggested that these two models actually are oversimplified and are biased in opposite directions: in that simple (non-cross-reacting) antibodies are assumed in the simple-complex model, while in the other, simple antigens (with only one kind of antigenic determinants) are assumed. Consequently both models are "restricted and complementary". Traditionally the HL-A and H-2 systems have been explained on the basis of the complex-simple and the simple-complex models respectively. Recently, however, Thorsby (1971) and Snell et al. (1971) independently proposed the alternate model for the H-2 system. Thorsby suggested that H-2 antigens can be arranged into two segregant series, corresponding to the specificities of the D and K end respectively, and that each H-2 allele from the two series determines only one antigen, the other H-2 spcificities associated with each allele would be caused by cross-reacting

antibodies and, thus, would represent mere serological artifacts.

"Public" and "Private" Specificities: H-2 specificities can be classified broadly into the so-called "public" and "private" ones (Snell et al., 1971; Klein and Shreffler, 1971). Public specificities are widely distributed among alleles, such as specificities 27, 28, 29; others may occur in only one or two types such as 17 and 33 and are called private specificities. In general, private specificities are more sharply defined and induce the production of high titred antisera. On the other hand, public specificities are less sharply defined and relatively weak. One further important distinction between these two categories is that private specificities are restricted either to the K or the D region, but not both; while public ones can be found in both, such as 3, 5 and 35. Snell et al. (1971) claimed that they were able to group the private specificities into two mutually exclusive or allelic series and suggested that these two H-2 series are the true homologs of the two H-2 series. The "non-private" or public ones, in their view, perhaps correspond to the yet undetected weak HL-A specificities.

It is pertinent to note that these private specificities were detected not by the conventional haemagglutination test but by the

the lymphocytotoxic test as for the HL-A system. If this is an intrinsic, rather than a coincidental finding, this piece of evidence may indeed be a strong foothold for establishing true homology of the two systems.

THE ROLES OF HUMORAL ANTIBODY AND CELL-MEDIATED IMMUNITY IN ALLOGRAFT REJECTION

The immunological nature of homograft rejection has been definitely established (Billingham et al., 1956; Brent, 1958). Two types of response can be obtained as a result of tissue transplantation: cell-mediated immunity (CMI) and humoral antibody production (HAP). Although it is generally agreed that CMI is primarily responsible for homograft rejection, yet a series of observations should bring out the importance of HAP as well.

The Role of Humoral Antibody: The classic studies of G.H. Algire in the 1950's provided strong evidence against the role of humoral antibodies in homograft immunity. Fetal mouse lung or skin epithelium was put inside a diffusion chamber, communicating with the outside only through the minute pores of the millipore membranes encasing the chamber, which allowed only the passage of macromolecules, e.g., antibodies, but not of cells. The chamber was then introduced into the

peritoneal cavity of an allogeneic host. Even if the recipient had been previously specifically sensitized, the homograft inside the chamber underwent destruction only if the pore size was large enough to allow the entry of host leukocytes (Algire, 1954; Algire et al., 1954, 1957). It was concluded, therefore, that antibody alone could not bring about graft rejection and that cells were required for this process. However some evidence against this view was provided by the work of Amos and Wakefield (1958, 1959) and Wakefield and Amos (1958) who pointed out that antibodies and C molecules do not readily pass through the pores of the millipore membrane and if antibody and C levels are high enough inside the chamber, graft destruction would result. Thus, Gabourel (1961) showed that allogeneic mouse fibroblasts could be so destroyed, both in actively and passively hyperimmunized hosts. Similarly Najarian and Feldman (1962) and Kretschmer and Perez-Tamayo (1962) independently reported that immune lymphoid cells placed inside diffusion chambers implanted subcutaneously or intraperitoneally into nonsensitized syngeneic or allogeneic hosts produced some diffusible substance that caused destruction of skin homografts on the body surface (though transplantation immunity could not be furnished with immune serum alone).

As in the case of experiments with diffusion chambers, early experiments with fetal lambs also provided evidence against the role of humoral antibody in allograft rejection, since fetal lambs which were thought to be agammaglobulinemic at this stage of maturation were capable of undergoing allograft rejection (Schinkel & Ferguson, 1953). However, it was later found that normal fetal lambs and calves, hitherto thought to be agammaglobulinemic, can indeed develop plasma cells and humoral antibodies at that stage of development against foreign antigens (Fennestad and Borg-Petersen, 1962; Silverstein et al., 1963), thus reviving the possible role of humoral antibody in the skin-allograft rejection phenomenon as reported by Schinkel and Ferguson (1953).

In relation to different types of grafts, the evidence concerning the role of humoral antibody in rejection may be summarized as follows:

(a) Skin Graft Rejection: In rabbits, Stetson has demonstrated accelerated destruction of established test skin allografts by passive transfer of immunity with serum from specifically hyperimmunized animals. However, he was successful only when the serum was administered locally (Stetson, 1959 a, b). Kretschmer and Perez-Tamayo (1962)

also reported that if cells sensitized against homografts were placed within diffusion chambers and embedded subcutaneously in host rabbits, the specific allogeneic skin graft on the host was rejected in an accelerated fashion as compared with the rejection time of an adjacent control skin graft. Steinmuller (1962) showed that serum obtained from BN rats at approximately the time of rejection of Lewis skin grafts (first-set) was consistently effective in producing accelerated rejection of test Lewis grafts in BN recipients. Sera collected at other times were less effective. It should be noted however that the capacity of the sera to induce accelerated rejection had no apparent relationships with their in vitro cytotoxic and haemagglutinating antibody levels.

It is interesting to note that in birds, similar findings have been reported. Hasek et al. (1961) and Haskova et al. (1962) found that large amounts of alloantisera were able to cause rejection of skin allografts in tolerant ducks upon passive transfer. Established skin allografts developed lesions within a few hours and were totally destroyed within one to two days.

(b) Rejection of Primarily Vascularized Organs: Relatively large body of evidence for the possible role of humoral antibody in

participation of graft rejection comes from renal transplantation (Kissmeyer-Nielson et al., 1966; Najarian and Foker, 1969; Porter, 1967; Jeannet et al., 1970). Lower (1969) also reported a heart case in which the serum of the graft recipient contained antibodies reactive with donor tissues.

Simonsen (1953) first observed an associated antibody response with rejection of allografted dog kidneys. The antiglobulin consumption method has provided evidence for antibody being present in the serum of virtually all patients after transplantation. When immune serum was passively administered, specific antibodies were promptly found in the kidney graft vasculature (Najarian and Perper, 1967). The most important finding giving unequivocal evidence for the participation of antibody in kidney graft rejection was that by Clark et al. (1968). A kidney was temporarily grafted into a sensitized but severely leukopenic dog and then returned to the original donor. Prompt (12-24 hours) rejection in the donor, where all cells are necessarily autologous, resulted.

(c) Immunity to Ascites Tumors: Snell (1957) has classified transplantable tumors into three groups: those that are susceptible to cytotoxic alloantibodies; those that are resistant, and those that

have intermediate sensitivity. Winn (1962) pointed out that it is the cell type that determines the degree of susceptibility. Tumor cells of lymphoid or bone marrow (myeloid) origins are particularly susceptible to humoral factors, while those from other tissues are less susceptible. For example, sarcomas, a connective tissue tumor, were found to be almost completely resistant. G. Moller, on the other hand, showed that the concentration of antigen receptors on the cell surface plays an important role in determining susceptibility to antibody, while a high concentration entails susceptibility, a low concentration would mean resistance (Moller, G., 1963).

(d) Immunologic Enhancement: In relation to (particularly) tumor transplantation, the phenomenon of immunologic enhancement has been noted in the past twenty years. In a sense, immunologic enhancement is a paradox to the usual conception that humoral antibodies in actively or passively immunized hosts would be expected to react against a foreign graft. However, the presence of antibody frequently leads to a delay in rejection of, say, a tumor, and which would grow to an extent that results in the death of the "immune" hosts. This is the phenomenon of immunologic enhancement (Kaliss and Byrant, 1956; Kaliss, 1957, 1962). Nearly all of the early studies on enhancement

were carried out with tumors. However, Billingham et al. (1956) reported immunologic enhancement in skin allograft, although prolongation was a matter of a few days rather than weeks as in the tumor case. In this direction, Winn (1970) was also able to extend survival times of skin grafts by passive transfer of alloantiserum.

It has been found that the lymphocytes from the hosts carrying the 'enhanced' tumors are cytotoxic for the tumor cells in vitro (Old and Boyse, 1964; Hellstrom and Hellstrom, 1969, 1970). Strong evidence was available suggesting that sera from the tumor-bearing animals are capable of blocking the cytotoxic effect of the specifically immune lymphocytes (Hellstrom and Hellstrom, 1970).

The Role of CMI: As noted above, the problem of the role of humoral antibodies in transplantation immunity is elusive and ill-defined. This would imply that serum antibodies may not be the principle mediators in transplantation immunity.

The first evidence that homograft immunity is cell-mediated came with the findings that while serum from an immune animal does not passively transfer immunity to tumor homografts in mice (Billingham et al., 1954), it was transferrable by means of living immune lymphocytes (Mitchison, 1955). The argument that plasma cells present in the

transferred cell population are responsible for the production of "molecules of immunity" which are actually responsible for the rejection phenomenon is hardly justified in view of the relatively small amount of antibody that can be produced by the transferred antibody producing cells.

Another strong piece of evidence was provided by Billingham et al. (1962) who showed that regional lymph node cells from either normal or specifically sensitized syngeneic animals, when transferred to an immunologically tolerant mice (or rats) bearing hitherto healthy, established skin grafts could cause rejection of the latter. Cells from sensitized animals were found to be more effective than cells from normal animals.

Transplantation immunity can also be manifested as delayed cutaneous inflammatory reactions. Brent, Brown and Medawar (1959) showed that guinea pigs sensitized by skin homografts respond to subsequent intradermal challenge with living donor cells from the homografts, or antigen extract prepared therefrom, with a local dermal inflammatory response of delayed origin -- the delayed hypersensitivity reaction -- very similar to the classic tuberculin reaction.

IN VITRO METHODS TO STUDY THE HISTOCOMPATIBILITY NATURE OF REJECTION

Both *in vitro* and *in vivo* methods have been developed to study histocompatibility. The following section will deal with only *in vitro* methods.

Cell-Mediated Immunity: Three *in vitro* reactions are at present recognized as being characteristic of CMI: a) the cytotoxic action of sensitized lymphocytes for target cells from the donor of the immunizing tissues, b) inhibition of migration of macrophages of peritoneal exudates from sensitized animals in the presence of specific antigen, and c) the mixed leukocyte reaction (MLR).

(a) Cytotoxicity of Lymphocytes: The cytotoxic reaction has been successfully used in the study of CMI induced by transplantation antigens. Sensitized lymphocytes of allograft recipients were shown to rapidly lyse target cells *in vitro* (Brunner et al., 1968). The pioneer studies on target cell lysis by sensitized lymphocytes as a model of CMI in transplantation immunity were undertaken by Govaerts (1960) who showed that thoracic duct lymphocytes from dogs sensitized by a kidney allograft were cytotoxic for cultured donor kidney cells in a 24-48 hours incubation period at 37°C. Antibodies and C were not required in this cell-killing process. Soon following this finding,

Rosenau and Moon (1961) and Wilson (1963) reported their success in showing that spleen and lymph node cells from sensitized mice or rats were cytotoxic for allogeneic target cells without added immune serum or C. The cytotoxic reaction has been shown to be specific. Lymphocytes sensitized to a "third party" transplantation antigen (Brondz, 1964), or to heterologous antigens (Wilson and Wecker, 1966) are completely ineffective. Also, specifically sensitized lymphocytes have no adverse effect on syngeneic target cells (Wilson, 1963; Brondz, 1964).

Cytotoxicity methods are numerous and are preferentially employed according to the convenience and interest of the investigators. Dead cells are differentiated from living ones by the ability of the latter to exclude supravital dyes, thus remaining unstained, while dead cells can readily take up the stain (Wilson, 1963, 1965). In this way percent dead cells (degree of cytotoxicity) can be calculated by direct enumeration. Though quantitative yet this method is laborious and involves a large degree of error.

Granger and Weiser (1964) and Moller and Moller (1965) used a monolayer plaque technique to assess the degree of cytotoxicity -- local reduction of target cell density (plaque formation) is looked

for and can be scored arbitrarily as grades of intensity. However, the long incubation time often required for plaque formation discourages its use as a routine assay.

A more objective and statistically valid method employs the release of radioactive labels, e.g. ^{14}C-thymidine (Vainio et al., 1964), ^{32}P-phosphate (Perlmann and Broberger, 1963) or ^{51}Cr-chromate (Brunner et al., 1968) from killed cells into the supernatant as the index of cytotoxicity. Of all these radioactive markers, ^{51}Cr is most favorably employed in labeling target cells because 1) once bound to cells, the isotope is not reutilizable in its reduced form, 2) most radioactivity is released from the dead cells, 3) labeling is rather stable with minimal spontaneous release from tissue cells and 4) efficient labeling can be achieved without laborious handling. In addition the desirable precision and reproducibility of the method further justify its being favored in both cell-mediated and antibody-mediated cytotoxicity assays.

(b) Inhibition of Macrophage Migration: George and Vaughan (1962) extended the observation by Rich and Lewis (1932) that explants of spleens and lymph nodes from tuberculin sensitized animals grow out poorly in the presence of tuberculin, but do quite well in its

absence, and developed the in vitro macrophage migration as a model for delayed hypersensitivity. They observed that in the presence of PPD (purified protein derivative), but not in the presence of other antigens, peritoneal exudate cells from animals sensitized to PPD failed to migrate normally. David et al. (1964) showed that a 2.5% concentration of "sensitized" cells in a population of otherwise normal peritoneal cells in cultures with antigen resulted in an inhibition of all cells. The "sensitized" cells were shown to be lymphocytes (Bloom and Bennett, 1966).

This migration inhibition technique has been successfully applied to transplantation immunity by Al-Askari and his collaborators (1965) and furthered by Lake et al. (1971). The basic principle of this assay is that peritoneal exudate cells from mice with homograft sensitivity are inhibited when mixed with peritoneal cells obtained from the strain of mice that had donated the sensitizing skin homografts. Peritoneal cells from unrelated strains do not influence each other. The reaction was thus shown to be specific.

The inhibition of migration assay has been refined and adapted for the quantitative study of transplantation immunity in vitro (Lake, 1972; Lake et al., 1971).

(c) The Mixed Leukocyte Reaction (MLR): The related phenomenon of blastogenesis per se refers to the transformation of leukocytes into blastoid and dividing cells. It can be initiated by either non-specific (e.g. phytohemagglutinin and other plant mitogens) or specific (specific antigens) means. Mills (1965) showed that lymphocytes from guinea pigs sensitized subcutaneously with tuberculin and other protein antigens in Freund's adjuvant will undergo a characteristic morphologic transformation upon exposure to the sensitizing antigen in tissue culture. This phenomenon is actually a secondary immunologic response at the cellular level (Wilson and Billingham, 1967).

This phenomenon was applied to transplantation immunity studies, in the mixed leukocyte reactions (MLR) which may be employed as a "two-ways" reaction, i.e. when the leukocytes of two unrelated individuals are mixed in vitro and react against each other, or as a "one-way" reaction when the leukocytes of one of the two individuals are treated with X-irradiation (Kasakura & Lowenstein, 1967) or with mitomycin C (Bach and Voynow, 1966) and are not capable of any reaction, but still provide the antigenic stimulus for the cells of the other individual to react. Bain and her colleagues (1963, 1964) first demonstrated that when peripheral blood leukocytes from two unrelated

individuals were mixed and cultured, blastogenesis took place and incorporation of radioactive thymidine resulted. They also observed the important fact that mixed culture of cells from monozygotic twins did not undergo transformation. Taken together, these observations showed that the peripheral leukocytes were able to mutually recognize histocompatibility antigens present on the cell surface. In the context of clinical transplantation it is particularly important to know whether the recipient immunocompetent cells would react against antigens present on the donor graft. The one-way MLR in which the donor cells are rendered inactive by antimetabolites such as mitomycin C has been designed for this purpose (Bach and Voynow, 1966). Two-ways and one-way MLR have been successfully applied in mice (Dutton, 1965, 1966); rats (Dutton, 1965; Wilson et al., 1967) and rabbits (Wilson, 1967; Chapman and Dutton, 1965). This kind of assay, however, takes a long incubation time (in the order of days). It is, therefore, not only time-consuming, but also easy to get contamination.

Methods for the Study of Antibody Producing Cells: It is possible to detect antibodies as they are in the serum (serum level), or as they are just released from the cell synthesizing them (cellular level). Only methods pertaining to the latter category will be discussed.

Several methods have been designed to detect antibody

production at the cellular level: the anti-flagellar antibody immobilization method of Nossal and Lederberg (1958), the fluorescent antibody technique of Vazquez (1961), the phage neutralization technique of Attardi et al. (1959), the localized hemolysin reaction of Jerne and Nordin (1963) and Ingraham and Bussard (1964), and the rosette technique of Zaalberg (1964). As is obvious, individual methods are suitable only in particular systems. Thus, in transplantation (histocompatibility) studies only the hemolytic plaque technique and the rosette technique have been used.

(a) Rosette ("Cluster") Technique: The original method as developed by Zaalberg (1964) was intended to detect anti-SRBC antibodies. Spleen cells from mice immunized with SRBC six days earlier were suspended in a test tube in 1 ml of tissue culture medium containing 1% washed SRBC. The test tubes were incubated for two hours at 37°C in a roller tube apparatus. The cells were then pipetted into a haemocytometer and counted. Complexes of at least five red cells attached onto the single spleen cells were arbitrarily scored as rosette forming cells.

Although both rosette and antibody plaque technique measure antibody producing cells, the kinds of antibody detected by these two methods are very likely to be different. The rosette technique probably in addition to cells releasing hemagglutinating antibodies

detects other cells which carry antibody-like receptors but do not secrete antibodies while the agar plaque technique deals with truly cytolytic antibodies that kill cells in the presence of C. Thus, Zaalberg et al. (1966) had tried to lyse the 'rosetting' cells by the addition of C. They found that the rosettes remained intact after incubation at 37°C, indicating that the 'rosetting' antibodies are non-hemolytic antibodies. These results, as pointed out by the authors, seemed to point to the fact that rosette formation and hemolytic plaque formation are actually manifestations of two distinct phenomena. These findings were supported by observations by Talmage et al. (1956a, b) and Stelos and Talmage (1957).

(b) Hemolytic Plaque Technique: The plaque technique as described by Jerne and Nordin (1963) is carried out in Petri dishes with a bottom layer of agar serving as a supporting, moist, translucent, yet non-nutritious medium. An agar solution is meanwhile maintained at about 42°C (to prevent solidifying) when a SRBC suspension, and a spleen cell suspension in which the antibody-producing cells against SRBC are being assayed, are sequentially mixed with the agar and then poured on top of the bottom layer in the Petri dishes. The agarose is then allowed to solidify (about 10 minutes) before incubation at 37°C for one hour. During incubation the antibody

produced by cells in a phase of active antibody production will diffuse through the agarose and sensitize the red cells in the immediate vicinity of the lymphoid cells. After this incubation fresh guinea pig serum as a source of C is added at a dilution of 1/10 into the dishes. They are re-introduced into the incubation chamber and incubated further for 30 minutes. During this second period of incubation the red cells which had previously become sensitized with antibody are lysed by the hemolytic complement. Plaques will appear as round clearings of about 0.7 mm in diameter in an even lawn of RBC's. A microscope will reveal that each plaque contains a lymphoid cell at the centre. These cells are usually referred to as plaque forming cells (PFC) and presumably are the cells which secrete antibody molecules into the immediate vicinity, i.e., they are antibody forming cells. Some observations support this role of antibody production by PFC. Firstly, the release of antibodies from PFC is temperature dependent, i.e., is stopped at $4^{\circ}C$ and resumes without a lag when the cells are transferred from $4^{\circ}C$ to $37^{\circ}C$, thus indicating active synthesis of antibody, rather than passive release of previously adsorbed antibody. Secondly, larger plaques are obtained with smaller number of target red cells. Thirdly, most PFC usually belong to the plasma cell series. Fourthly, the number of plaques is directly

proportional to the number of lymphoid cells plated. Taken together these can be interpreted that this hemolytic plaque technique measures true antibody producing cells in the spleen cell population.

In retrospect the afore-mentioned authors then were not aware of the fact that their technique which employed GPC only, did not (and were not able to) detect cells producing antibody with low hemolytic efficiency (7S) (Rowley and Fitch, 1964; Bussard and Binet, 1965). In 1965, Humphrey demonstrated that 19S antibodies were more effective in inducing hemolysis than 7S antibodies. On the other hand, Riha (1964) showed that the hemolytic activity of 7S antibodies could be increased by using an antiserum specific for the 7S antibodies. Prompted by these findings Sterzl and Riha (1965) and Dresser and Wortis (1965) independently demonstrated the use of anti-gamma G antiserum to facilitate 7S plaque formation. The antiserum was always prepared by immunization of a xenogeneic animal with the gamma G from the species the PFC of which were to be assayed. For example in a mouse anti-SRBC system, a rabbit anti-mouse gamma G antiserum was used as the developing antiserum. Sterzl and Riha (1965) found that there was an optimal concentration of the developing antiserum for maximal development of plaques, and higher concentration resulted in inhibition of 7S plaques and to some extent, 19S plaques

also, presumably because of the presence of antibodies directed to the light chain, common to both 7S and 19S molecules.

Humphrey and Dourmashkin (1965) and Bor made a mathematical estimation as to the number of 19S and 7S antibody molecules required to produce a functional lesion on the cell membrane to bring about its death. They came to the conclusion that only a single 19S antibody molecule is enough to effect a lesion while two adjacent 7S molecules on the cell surface are required. Statistically, this is not a frequent happening, and for this reason, high concentration of 7S antibody or an external aid in the form of anti-7S antibody is required. Effectively the specific attachment of anti-gamma G antibodies onto gamma G antibody molecules mimics the case in which two identical gamma G molecules are sitting side by side, thus capable of producing a functional lesion upon addition of C. The anti-gamma G antibodies impart a "piggy-back" effect to the system, as illustrated by the picture depicted by Dresser and Wortis (1965).

BRIEF REVIEW OF STUDIES ON ANTIBODY PRODUCING CELLS IN TRANSPLANTATION IMMUNITY

Antibody production at the cellular level in transplantation immunity has been studied by methods originally designed for heterologous (xenogeneic) systems, usually anti-SRBC systems. The methods can be divided into two categories, viz., the plaque and the rosette ("cluster") techniques mentioned earlier.

Rosette ("Cluster") Technique: Modification of the original technique by Zaalberg (1964) for use in histocompatibility studies was made by Micklem et al. (1970). CBA mice were skin-grafted with strain A donors. In a period from day 4 to day 27 after grafting, brachial and axillary lymph nodes were removed and single cell suspensions prepared. The lymph node cells were mixed with donor RBC in the ratio of 2:5 respectively. Concentration was adjusted to 2.1×10^7 per ml, in one ml. This was left overnight at $6^{\circ}C$. Half of the supernatant was taken off. Cells were then resuspended by end-over-end rotation for 2 minutes and the clusters counted.

In this way they found that an exponential increase of cells forming clusters with allogeneic erythrocytes between 4 - 12 days, followed by a slow return toward the background level from the

14th day onward. It should be noted that there was a short duration of an increase in number of cluster-forming cells formed with syngeneic red cells, in contrast with those formed with allogeneic red cells. This finding is not completely in agreement with the idea that cluster-forming cells have a direct involvement in homograft rejection, though it by no means rules out the possibility.

Micklem et al. (1971) also studied the second-set response to allografts in mice. They found that on the third and subsequent days most of the mice after the second graft had specific RFC above the control range, but they did not show a progressive rise such as characterized in the first-set response. Since it has been shown that in both the primary and secondary responses serum antibody levels had similar kinetics (Micklem and Brown, 1966), the authors suggested that the lack of correlations between the kinetics of RFC formation and serum antibody production implies that RFC are not the main secretors of antibody, and that these cells might be just antigen recognition cells.

Agar Plaque Technique: Hildemann and Pinkerton (1966) first attempted to modify Jerne's localized hemolysis in gel in the studies of transplantation immunity. They posed the question of whether early appearing

macroglobulins (19S) are involved in allograft rejection. Plaque forming cells in spleens are enumerated after a single intravenous immunization with allogeneic blood cells in different donor-recipient combinations as shown in the following table. It can be seen that the

Summary of Mouse Spleen Cell Plaque Production in Relation to Strong and Weak Histoincompatibilities

Donor strain	Recipient strain	Histocompatibility locus difference between donors and recipients	Skin allograft median survival times in days with range of rejection times in parentheses	Average plaque count per 10×10^6 spleen cells at peak primary response*	Peak plaque production	Range of primary plaque production	Average plaque count per 10×10^6 spleen cells from non-immunized control mice*
					day	*days*	
A/J	C57BL/6	H-2 plus Non-H-2	8.2 ± 0.5‡ (7–10)	26.6 (29.6)	6	4–12	3.6 (4.0)
B10.A	C57BL/10	H-2	10.3 ± 1.2§ (9–14)	15.6 (17.4)	5	3–12	1.2 (1.3)
C57BL/10	B10.A	H-2	10.8 ± 1.5§ (9–14)	9.6 (10.7)	5	3–12	1.1 (1.2)
C57BL/6♂	C57BL/6♀	H-Y	25.0 ± 2.5‖ (14–75)	15.1 (16.8)	9	4–13	1.0 (1.1)
C3H.K	C3H	H-1	29¶ (16–43)	14.8 (16.4)	10	6–13	1.5 (1.7)
C3H	C3H.K	H-1	91¶ (35–124)	3.3 (3.7)	9	8–12	1.6 (1.8)

peak of plaque production occurred generally earlier and reached higher levels in combinations involving stronger H-2 histocompatibility barriers, as compared with combinations involving weaker H-1, or H-Y antigenic barriers. However, there was no correlation between the time of appearance of the peak of plaque production and the median survival

times in the weak combinations. In light of these findings they concluded that early 19S antibodies do not appear to be directly involved in allograft rejection.

The group of the State University of New York at Buffalo (Fuji et al., 1971) further modified the plaque technique by employing allogeneic normal nucleated cells as target instead of the red cells employed by Hildemann and Pinkerton (1966). Also they used rabbit C instead of the more conventional GPC as used by Hildemann and Pinkerton. While Fuji, Schultz and Milgrom (1970) studied a xenogeneic system (mouse anti-rat), Fuji, Zaleski and Milgrom (1971) employed a mouse allogeneic system using thymus and tissue cultured lymphoblast as targets. They made an interesting observation that thymus but not lymphoblast target cells are capable of detecting anti-θ-producing PFC, whereas lymphoblasts but not thymus target cells are capable of detecting anti-H-2 producing PFC. Another interesting finding was that although spleen cells and lymph nodes cells are known to be rich in H-2 antigens, anti-H-2 responses could not be detected with these cells as targets. In addition to immunization with nucleated allogeneic cells injected intravenously, skin allografts were also found to be effective, showing that transplantation antigens per se are responsible for the appearance of PFC.

Nordin, Cerottini and Brunner (1971) also reported their success of their use of mastocytoma P-815-X2, a tumor propagated in the DBA/2 ($H\text{-}2^d$) strain, as the source of target cells. Again rabbit C was used, in fact, they could not substitute it with GPC. In this way, a peak PFC response was detected on the 10th day with about 7,000 PFC per spleen. They also studied the importance of the number of H-2 specificities involved in the individual systems in revealing PFC. Their results showed that as long as the target cells contain the antigen specificities against which the PFC are producing antibodies, the magnitude of the PFC response remains the same, regardless of the number of specificities involved. In other words, it is an all-or-none phenomenon, at least in these authors' hands.

To summarize, two kinds of target cells have been used in the agar plaque technique: red blood cells (Hildemann and Pinkerton, 1966) and nucleated cells. The latter may be normal lymphoid cells or a lymphoblastic cell line cultured *in vitro* (Fuji et al., 1971), or neoplastic cells propagated *in vivo* (Nordin et al., 1971). The relative merits of using these cells as targets will be discussed in due course (page 41).

RATIONALE OF THE PRESENT INVESTIGATION

As may be seen from the above review of the most important data of the literature, there is still very little knowledge about the cells involved in allograft rejection. On the one hand, the role of CMI is pretty well delineated as the main mechanism for allograft rejection. However, it is not yet clear which cells are responsible for the complex phenomenon which is generally known as CMI. Long-lived, thymus dependent lymphocytes of the recirculating pool have been implicated as the cell type responsible for the recognition of transplantation alloantigens (Miller and Osoba, 1967) and, therefore, for the initiation of the immune response against the allograft. However, other cell types also are almost certainly involved in allograft rejection. Thus macrophages have been shown to be capable of specifically killing target cells in vitro (Granger and Weiser, 1964, 1966) and are largely represented in vivo in the cell populations that infiltrate allografts during rejection. Short-lived non-recirculating lymphocytes have also been implicated in cell-mediated immune phenomena mainly in relation to antibacterial defense mechanisms (MacKaness and Blanden, 1967) and may play some role in allograft rejection too.

On the other hand, the role of humoral antibody, and of the cells which produce such antibody, is at least as complex and as obscure as that of CMI. Alloantibodies have been demonstrated to have at least two opposite effects in relation to graft rejection, i.e., enhancement and damage of the graft. Enhancement is probably a complex phenomenon which may be mediated by afferent, efferent or central blockage of the immune response, and the respective role of these different mechanisms may vary according to different conditions. Damage of the graft by humoral antibody may also be due to several mechanisms, such as direct cytotoxicity of C activated by the Ag-Ab reaction on the cell surface (Gorer and O'Gorman, 1956; Brunner et al., 1970); indirect effects of C through chemotactic factors, liberated during its activation, which induce accumulation of polymorphonuclear cells and of the phagocytes and even to the conditions for digestion of the grafted tissues (Gewurz et al., 1968), activation of cytotoxic lymphocytes by complement on the cell surface (Gotze and Muller-Eberhard, 1970), or by Ag-Ab complexes on the cell surface in the presence of unsensitized lymphocytes but in the absence of added C (Holm and Perlmann, 1967; Perlmann et al., 1968), etc.

In order to unravel such extreme complexity of mechanisms a study of the cells which are activated during allograft reaction

appears essential. Within the framework of such a long term study, the present investigation was specifically aimed at identifying the cells which produce antibodies against transplantation alloantigens at the cellular level.

As noted previously there are two assays for such studies in transplantation immunity: the agar plaque technique using different kinds of cells as target (indicator) cells (Hildemann and Pinkerton, 1966; Fuji et al., 1971; Nordin et al., 1971), and the rosette (cluster) technique (Micklem et al., 1970). Although the rosette technique has the advantage of being a technically simple and rapid assay, there is still some controversy about the real nature of the cells detected by this technique. Thus, as Storb and Weiser (1968) pointed out, the rosette technique detects not only lymphocytes, or plasma cells, but also macrophages and reticulum cells which may have adsorbed cytophilic antibodies. On the other hand, plaque forming cells have been shown to belong to the lymphocytic lines that are proven to be antibody-producing cells (Harris et al., 1967). Moreover, while the hemolytic plaque technique detects true hemolytic antibodies, the most probable kind of antibody produced by RFC is haemagglutinins. Both these types of antibody are probably important in the development of transplantation immunity, but cytotoxic (hemolytic) antibodies are more likely to

be involved in rejection, and non-cytotoxic antibodies (agglutinating) are probably more important for enhancement.

In strong xenogeneic systems, e.g., rabbit anti-SRBC or mouse anti-SRBC, the agar plaque technique as originally developed by Jerne and Nordin (1963) served as a reasonably satisfactory tool to detect such immunologically committed cells. In much weaker intraspecific (allogeneic) systems, much difficulty had been encountered with the original plaque technique. The report by Hildemann and Pinkerton (1966) has made this clear. The methods described by Fuji et al. (1971) and by Nordin et al. (1971) are restricted in their applicability, since they call for the use of established lymphoblastic culture lines or neoplastic cells respectively as target cells. Therefore these methods can be applied only to strain combinations for which either lymphoblastic cell lines or neoplasms sensitive to cytotoxic alloantibodies are available. This represents a severe limitation of the applicability of these methods since most neoplastic cells are resistant to cytotoxic antibody (Moller, G., 1963; Kaliss and Byrant, 1956; Kaliss, 1957, 1962) while the establishment of new cell lines is a difficult and laborious task. As will be discussed later, the technique described in this thesis may find application in the study of the specificity of immunity to allografts, which requires the use of target

cells from many different strains, all having similar sensitivity to alloantibodies. On the other hand, the advantages of using RBC as targets are numerous. Firstly they are easily available from any strain of mice, and requiring no special manipulation to obtain good samples. Secondly they are very sensitive to lytic antibodies. Thirdly they carry most of the H-2 and some non-H-2 specificities (Snell and Stimpfling, 1966). However, the method of Hildemann and Pinkerton (1966) is not very sensitive and was found to be very difficult to reproduce (see page 82). Therefore, it was decided to make an attempt to develop a more sensitive technique with the use of RBC as targets to study antibody producing cells in transplantation immunity.

With this in mind pilot experiments were undertaken to determine the best conditions for such a method. Towards this direction physical variables were considered first, such as the thickness of the top agarose layer, the concentration of the target red cells and of the immune lymphoid cells, etc. The problem was next approached with biological means. Rabbit and guinea pig complement were compared for their hemolytic activity in the present system. A rabbit anti-mouse RBC was produced and tested for its ability to increase the number of visible plaques. Neuraminidase (VCN) (Sanford, 1967; Schlesinger and Amos, 1971) was also tested for the same purpose. A rabbit anti-mouse

gamma G immunoglobulin antiserum was also produced and employed, in conjunction with Concanavalin A (Nordin et al., 1969), to investigate the antibody classes involved in the present system.

It will be seen that such a hemolytic plaque technique as described was applicable to studies of the mouse H-2 system not yet attainable by other methods.

MATERIAL AND METHODS

Chemical and Biochemical Materials: Radioactive chromium (^{51}Cr) was supplied by Atomic Energy of Canada Ltd., Ottawa, Ontario, in the form of Na_2CrO_4 in NaOH solution with specific activity 80 - 150 µci/gm. Before use, it was neutralized with 0.2N HCl and made up to a concentration of 1000 µci/ml with Dulbecco Solution. Radioactivity was counted in a well-type gamma scintillation counter (Nuclear Chicago Corp., Des Plaines, Illinois).

Agarose (SeaKem) was obtained from Marine Colloid, Inc., U.S.A.

Concanavalin A was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Neuraminidase prepared from Vibrio cholerae (VCN) was purchased from General Biochemicals, Ohio.

α-CH_3-D-Mannoside (Sigma Chemical) was a gift from Dr. Goldenberg, Manitoba Cancer Research Foundation, Winnipeg.

EDTA (Ethylenediaminetetraacetate) was supplied by Matheson Coleman and Bell Co., Ohio.

Petri Dishes: Sterile plastic Petri dishes (60 x 15 mm) were obtained from Falcon Plastics, Dickinson and Company, Maryland, U.S.A.

Tissue Culture Plate: MicroTest II tissue culture plates were obtained from Falcon Plastics, Dickenson and Company, Maryland.

Plate Sealer: Microtiter plate sealers were supplied by the Cooke Engineering Co., Virginia.

Fetal Calf Serum: Fetal Calf Serum, immunoelectrophoresis precipitation tested (IPT), was purchased from GIBCO, Grand Island, N.Y.

Tissue Culture Media: Hanks Balanced Salt Solution (Hanks and Wallace, 1949) was prepared by reconstituting the dehydrated salt supplied by BBL, Division of BioQuest, Maryland. 0.01M N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Calbiochem) (Shipman, 1969) was used in place of the conventional sodium bicarbonate as the buffering agent. The pH was adjusted to 7.1. This solution will be referred to as HEPES-Hanks.

MEM (Minimun Essential Medium) Spinner Salt Solution was prepared from the 10-fold concentrated MEM Spinner Salt Solution supplied by BBL, Division of BioQuest.

Dulbecco Solution (Dulbecco and Vogt, 1954) was purchased from Difco Laboratories, Detroit.

Animals: (a) Mice: The following inbred strains, all of age two to three months, which are reported with their respective H-2 genotypes,

were purchased from the Jackson Laboratory: A/J (abbreviated A), H-2^a; C57BL/6J (C57BL/6), H-2^b; AKR/J (AKR), H-2^k; CBA/J (CBA), H-2^k; DBA/1J (DBA/1), H-2^q; DBA/2J (DBA/2), H-2^d; C3H/HeJ (C3H), H-2^k; and $B6AF_1$/J ($B6AF_1$, F_1 hybrids of A and C57BL/6), H-2^a/H-2^b.

The following congenic strains were also purchased from the Jackson Laboratory: B10/J (B10), H-2^b; B10.A/J (B10.A), H-2^a; B10.D2/J (B10.D2), H-2^d; B10.BR/J (B10.BR), H-2^k.

(b) Guinea Pigs and Rabbits: Outbred young adult guinea pigs and rabbits for supplying fresh sera as a source of C were obtained from the North American Laboratory Supplies, Winnipeg.

Complement: Guinea pig serum as a source of complement was prepared by bleeding an anaesthetized animal through cardiac puncture, and the blood collected in plastic test tubes. It was allowed to clot at room temperature for 15 minutes. After rimming the clot, the tube was refrigerated for a few hours at 4^oC in order to allow the clot to shrink. The test tubes were then centrifuged in a clinical centrifuge at 4^oC. The supernatant serum was divided into aliquots of suitable volumes and stored at -20^oC until use. The serum was never stored over three weeks.

Rabbit serum (RS) as a source of C was collected from the ear vein. The rest of the procedure was the same as for the guinea

pig serum. Just before use the RS was absorbed with agarose in order to reduce the natural anti-mouse antibodies (Cohen and Schlesinger, 1970), in the presence of EDTA (Boyse et al., 1970) (in order to avoid unnecessary loss of C). RS, diluted 1:4 with PBS, pH 7.2, and containing 1% 0.1M EDTA, was added to agarose (SeaKem) in the ratio of 3 ml to 0.1 gm. This mixture was kept in ice for 30 minutes with frequent mixing using a Pasteur pipette. The agarose was removed by centrifugation after absorption, and the serum was recalcified by the addition of 0.1M $CaCl_2$. Further dilution of this absorbed RS was made in ordinary HEPES-Hanks'.

Immunization Procedures:

(a) For the Production of Alloantisera:

(1) C57BL/6 anti-A: C57BL/6 animals received 1 x 10^7 SaI (indigenous to A strain) cells intraperitoneally the first time, and 3.5 x 10^7 cells intraperitoneally every week thereafter for eight weeks. The animals were bled 10 days after the last injection from the jugular vein. The antiserum was heated at 56°C for 30 min., and stored in 1 ml portions at -20°C.

(2) A anti-C57BL/6: Strain A mice were injected with 1 x 10^7 C57BL/6 lymphoid cells (from thymus, spleen and lymph nodes) intraperitoneally at the first instance, and 2.5 x 10^7 cells weekly

thereafter for seven weeks. The animals were given a month's rest, and then given weekly injections of 5×10^7 cells intraperitoneally for six more weeks. Blood was collected 10 days after the last injection from the jugular vein. The serum was stored in 1 ml portions at $-20^{\circ}C$ after heat inactivation at $56^{\circ}C$ for 30 min.

(b) For the Study of Plaque Forming Cells: Three months old C57BL/6 animals were given primary immunization by subcutaneous injection of 1×10^7 A SaI cells. This dose was divided equally on the two sides of the chest. An additional dose of 1×10^5 cells was given intraperitoneally at the same time. After three weeks, these animals were given a secondary immunization of an intraperitoneal injection of 4×10^7 A spleen cells. Spleens were then taken out by sacrificing the animals and the spleen cells were prepared as described elsewhere.

In experiments when mastocytoma cells were used as the immunogen, the procedure of Nordin et al. (1971) was followed. C3H mice were injected intraperitoneally with one single dosage of 30×10^6 DBA/2 mastocytoma cells contained in 1 ml of HEPES-Hanks'.

In the mouse anti-SRBC system, C57BL/6 mice were injected intraperitoneally once with 0.2 ml of 12.5% SRBC. Spleens were then taken out for assay at various days after the single immunization, as

will be described in due course.

(c) For the Production of Rabbit Anti-Mouse RBC Antiserum (RAMRBC): 1 ml of 25% mouse RBC in complete Freund's adjuvant (CFA) was injected at the rabbit's four foot-pads at day 0 and 7. Two weeks later, the same dosage of MRBC was administered subcutaneously with CFA. A final injection was given one week later with twice the dosage (1 ml of 50% RBC) with CFA. The rabbit was bled ten days after the last injection and the serum thus collected was heated at 56°C for 30 min. before storage in aliquots of 0.5 ml each at -20°C.

Labeling of Target Cells for Cytotoxic Tests: Radioactive chromium was added to a washed lymph node cell suspension containing 1×10^7 cells/ml to obtain a final concentration of radioactivity of 100 μCi/ml in a conical graduated tube (Pyrex). The mixture was incubated at 37°C in a water bath for 40 min. with constant agitation. The cells were then washed five times with HEPES-Hanks' and finally adjusted to 1×10^6/ml for use.

Labeled thymus cells were prepared in the same manner.

Titration of Alloantisera Using Cytotoxicity Test:

(a) C57BL/6 anti-A: Micro-tissue culture plates (Micro-Test II) with each well (flat-bottomed) holding a maximum of 0.3 ml was used. The alloantiserum was serially diluted in two-fold dilutions

in 0.1 ml HEPES-Hanks'. To each well, 0.1 ml of ^{51}Cr-labeled A lymph node cells (1×10^5 cells) was added using an Oxford Sampler. Normal C57BL/6 serum, heated at 56°C for 30 min. was used instead of the test serum in the control wells. The plate was allowed to stand at room temperature for 15 min. and 0.1 ml of ten-fold diluted GP serum was then added to each well, followed by an incubation at 37°C for 30 min. After incubation the plate was spun at 350 g for 4 min. 0.1 ml of the supernatant from each well was then carefully taken out with an Oxford Sampler and the radioactivity released in it was counted with a well-type gamma scintillation counter.

(b) A anti-C57BL/6: The procedure was exactly the same as in (a) except that, in this case, target cells were C57BL/6 lymph node cells.

(c) Calculation of Percent Lysis: Cytotoxicity was calculated according to the formula $L = \frac{T - C}{R_{100} - C} \times 100$, where L = percent lysis; R_{100} = 100% releasable radioactivity (counts) in the supernatant in the presence of antibody and C; T = counts in the supernatant in test wells, and C = counts in the supernatant in the presence of heated (56°C/30 min.) C and antiserum.

Cytotoxic Inhibition Test for Alloantigen: This test measures the

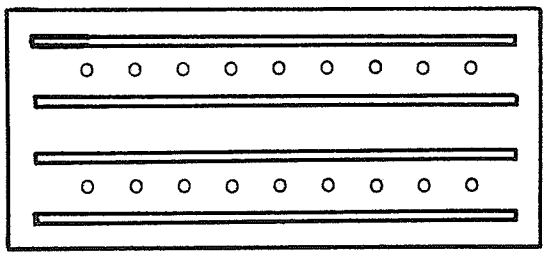
ability of an antigenic preparation to inhibit the cytotoxicity of an antiserum by first mixing the antigen and antiserum before the cytotoxic test. Again the micro-method using MicroTest II tissue culture plates was used. To a series of wells was added 0.1 ml of the cytotoxic serum at a dilution known to induce about 80% lysis. 0.1 ml of the test antigen in serial dilutions was added to each of these wells. The plate was allowed to stand for 15 min. 0.05 ml of the labeled target cell suspension (containing 1×10^5 cells) was then added to the wells and the plate further incubated for 10 min. at room temperature. 0.05 ml of 6-fold diluted GP serum was added as the C source. The plate was sealed with a plate sealer and incubated at $37^{\circ}C$ for 30 min. The rest of the procedure was the same as for the cytotoxicity test mentioned above.

Preparation of Rabbit Anti-Mouse Gamma G Antiserum (Developing Antiserum):

(a) Preparation of Mouse Gamma Globulins: Crude gamma globulins were first prepared from normal heat-inactivated ($56^{\circ}C$/30 min.) mouse serum by 50% $(NH_4)_2SO_4$ precipitation. The crude preparation was then partially purified by agar block electrophoresis as follows. An agar block (25 x 8 cm) was prepared by solidifying 300 ml of 0.85% of agar in barbital buffer, u = 0.05, pH = 8.6. A central agar

strip of 8 x 1 cm was cut out; the bottom of the trough thus formed was covered with a small amount of agar. The crude mouse gamma globulin sample, now mixed with an equal volume (5 ml) of 1.7% agar, was then introduced into the trough and allowed to set. After complete solidification a stable current (200V, 100 mA) was applied across the ends of the block and maintained for about 26 hours. After electrophoresis the agar block was cut into strips of 8 x 1 cm each, which were then individually introduced into 15 ml test tubes and submitted to freezing and thawing three times so as to release the serum proteins contained in them. The protein content of these fractions was then determined by reading the optical density at 280 mu (Beckman 1098).

In order to determine the distribution of gamma G protein in the different fractions, analytical immunodiffusion was done in the following manner. Agar slides (7.5 x 5.0 cm) were prepared with 1% agar in borate buffer of 0.005M and pH 8.0. Two sets consisting of a series of holes (1.5 mm in diameter) with a trough on each side were punched parallel to the major axis of the slide, as illustrated in the accompanying figure. The eluates from each of the strips



were introduced in a different hole. A rabbit anti-mouse gamma G_2 antiserum and a rabbit anti-mouse whole serum (MWS) were placed in the troughs for the identification of the gamma immunoglobulins contained in the fractions. The slides were incubated at room temperature for 24 hours in a moist chamber before observing for precipitin lines.

The individual eluates known to contain gamma G_2 were then pooled and concentrated by negative pressure dialysis. The purity of this concentrate was further checked by immunoelectrophoresis (barbital buffer, u = 0.05, pH 8.0).

(b) Production of Rabbit Anti-Mouse Gamma G Antiserum: About 320 ug N equivalent of the gamma G fraction was emulsified with an equal volume of complete Freund's adjuvant and injected into the four foot-pads of a young adult rabbit. Two weeks later, the rabbit was re-injected with the same amount of gamma G (i.e., 320 µg N), this time subcutaneously at multiple sites with CFA. The animal was test-bled nine days after the second immunization. Blood was collected on the 11th day by bleeding from the ear vein.

(c) Preparation of Kappa-Type Bence Jones Protein: Since both mouse IgM and IgG are known to possess K light chains (Potter, 1967), to render the developing antiserum specific for mouse gamma G, it is necessary to absorb out the anti-K (i.e., anti-kappa light chain)

antibodies which are very likely to be present together with anti-gamma chain antibodies.

Twenty femal BALB/c animals were inoculated with a MOPC 149 tumor (K-type myeloma) subcutaneously near the right arm pit. Urine from these animals was collected seven days later, three times a day for a period of twelve days. 0.01% sodium azide (NaN_3) was added as a preservative. After the last collection, the pooled urine was centrifuged at 10,000 rpm (Sorvall RC2-B, SS34 rotor) for 10 min. to remove any coarse debris, and then dialysed against distilled water for three days with at least two changes per day. The sample was then concentrated by dialysing it against 0.05M Tris-acetate buffer, pH 5.5, under negative pressure. The concentrated sample was applied to a DEAE-cellulose column equilibrated with the same buffer. The eluate was collected in 6 ml fractions. All of the above procedure was done in the cold at $4^{\circ}C$. Optical density of the fractions was read using a Beckman spectrophotometer 1098 and the protein concentration was calculated assuming the extinction coefficient for 1% concentration (i.e. 10 mg/ml) = 11.4 O.D. at a wavelength of 280 mμ. The fractions with significant O.D. were pooled and concentrated by dialyzing it against Tris-acetate buffer under negative pressure. The concentrate was stored at $4^{\circ}C$ before use.

(d) Determination of the Amount of Bence Jones Protein (BJP) for Absorption: The optimal amount of the Bence Jones protein for absorption of a known amount of the developing antiserum was determined by the two drop test. The BJP was diluted serially in 3 ml tissue-culture tubes (Pyrex) with a starting concentration of 120 ug N/ml total protein. Two drops of each dilution were transferred to individual micro-tissue-culture tubes with a clean Pasteur pipette. To each of the tubes, two drops of the undiluted developing antiserum were introduced (using another Pasteur pipette of a comparable bore size as the previous one) and immediately followed by gentle tapping of the tubes. The tubes were allowed to stand at room temperature for about 45 minutes. The tube showing maximal turbidity contains the optimal proportions of antigen and antibody. In this way, the desired amount of BJP for absorption of a certain volume of antiserum can be calculated (please see page 91).

(e) Absorption: The antiserum and BJP were mixed at the optimal ratio (determined as described above) in a 5 ml test tube and well stoppered. It was secured onto the rolling drum of a multi-purpose rotator (Scientific Industries) and rotated at a low speed. The antigen-antibody reaction was allowed to take place at room temperature for one hour, and then at $4^{\circ}C$ overnight. The precipitate was removed

by centrifuging at 15,000 rpm for 30 mins. (Sorvall RC2-B, SS 34 rotor). The absorbed developing antiserum was then stored in aliquots at -70°C.

Preparation of Soluble Alloantigens: The method used for extracting alloantigens from lymphoid cells was the hypertonic salt method described by Reisfeld et al. (1970) (see also Reisfeld and Kahan, 1971). Lymphoid cells from thymus, spleen and lymph nodes were prepared by teasing the organs with a pair of needles (attached to syringes). The cell suspension was filtered through an 80 mesh stainless steel screen and washed three times with HEPES-Hanks'. The cells was suspended in 3M KCl in HEPES-Hanks' (10 ml per 10^9 cells). The mixture was contained in a large test tube and rotated with a multi-purpose rotator at 4°C for 16 hours. The tube was then centrifuged at 130,000 g (Ultracentrifuge, Beckman L2-65) for two hours and the supernatant subjected to dialysis against HEPES-Hanks' under negative pressure with a total of 3 changes in two days. Protein concentration was determined by the Lowry method (Lowry et al., 1951). Extracts from different strains were prepared simultaneously (thus keeping the experimental conditions as constant as possible). The working materials were then stored at 4°C before use.

Preparation of Spleen Cell Suspension for Assay in the Plaque Technique:

Mice were killed by cervical dislocation and the spleens carefully removed and immersed into ice-cold HEPES-Hanks' supplemented with 20% fetal calf serum (heat-inactivated at 56°C for 30 minutes). They were then teased with two needles in order to release the cells. The crude suspension was filtered through a stainless steel 80 mesh screen. The suspension was allowed to stand in ice for about five minutes, letting the clumps settle. The clump-free suspension was then washed once with ice-cold HEPES-Hanks' (without FCS) and adjusted to the desired concentration. In this way, an 89-92% cell viability was constantly obtained, as determined with the trypan blue exclusion test.

Indicator (Target) Cells for Plaque Assays:

(a) SRBC: Fresh sheep blood (citrated) was supplied by the National Biological Laboratories, Winnipeg.

(b) MRBC: Mouse blood was collected afresh immediately before each experiment either from the tail vein or the jugular vein into Alsever's solution (Kabat and Mayer, 1961).

In either case red cells were prepared as follows: The blood was washed four times with HEPES-Hanks' by centrifuging at 250 g for three minutes. Each time the supernatant was aspirated off with clean Pasteur pipettes. Care was taken to remove the buffy coat

leukocytes usually layering at the top of the red cell pellet. The final concentration was adjusted to be 12.5% in HEPES-Hanks' unless otherwise stated.

In experiments using neuraminidase (VCN), mouse RBC were pretreated with the enzyme as follows: Two 0.5 ml portions of 12.5% mouse RBC in HEPES-Hanks' contained in siliconized 3 ml conical tissue culture tubes were mixed with 250 units (500 units/ml) and 50 units respectively. The final volumes in both cases were 1 ml. The control group contained HEPES-Hanks' without enzyme. These were incubated at 37°C for 30 minutes, during which time the tubes were shaken at frequent intervals. The MRBC were then washed three times to remove the enzyme with cold HEPES-Hanks' and finally adjusted to 12.5% in the medium. These preparations were to be incorporated into the top layer. The rest of the experiment was the same as described in the next section.

(c) Mastocytoma (P-815-X2) Cells: Mastocytoma, an ascitic tumor indigenous to DBA/2 ($H\text{-}2^d$), was propagated through successive transfers in the peritoneal cavity of DBA/2 mice. Tumor cells were harvested about one week after transplantation and were collected either by sacrificing the animal and opening the peritoneal cavity, or by putting the animal under ether anesthesia without sacrificing it and

aspirating the peritoneal fluid with a 20 gauge needle affixed onto a 5-ml plastic syringe. This peritoneal fluid was then diluted into MEM spinner medium and washed four times with the same medium. The use of other media was found to cause clumping of cells. The cells were finally counted in a haemocytometer and suspended in HEPES-Hanks'.

The Plaque Assay:

The haemolytic plaque technique was basically the same as that developed by Jerne and Nordin (1963). However, several significant modifications will be noted along the description of the technique.

(a) Preparation and Storage of Petri Dishes with an Agarose Bottom Layer: Sterile plastic petri dishes were layered with 2.5 ml of 0.83% agarose in phosphate buffered saline (PBS), pH 7.2. They were then stored in tight plastic boxes (to prevent loss of moisture) kept at $4^{o}C$.

(b) Pretreatment of Petri Dishes before Experiment: Just before an experiment, the desired number of Petri dishes were prewarmed in a $37^{o}C$ incubator. This pretreatment is necessary since a cold surface would instantly solidify the agarose top layer (described later) once it is introduced into the Petri dishes.

(c) Preparation of the Top Layer and Incubations: The preparation of the top layer is a critical step of the technique and

requires considerable empirical experiences and care.

Spleen cell suspension containing the alloantibody-producing cells to be assayed and the indicator (target) cells were prepared as described elsewhere. They were then allowed to stand to reach the room temperature. Meanwhile 12 x 75 mm plastic tissue culture tubes, each containing 0.3 ml of 2-fold concentrated HEPES-Hanks' were maintained at about 42°C in a water bath. To each of these, 0.3 ml of 0.83% agarose in double distilled water was delivered and mixed by gentle shaking, preferably with the liquid portion still immersed in the 42°C water to prevent partial gelling. A few minutes later, when the mixture had attained the temperature of the water bath, 0.1 ml of the target cell suspension and 0.1 ml of spleen cell suspension (2×10^8 ml) were sequentially added to each of two agarose tubes (more than two cannot be handled at one time). The final concentration of agarose was thus 0.31%. Mixing the contents at each addition is very important and should be done with the liquid portions immersed in the water bath to avoid premature solidification. The contents in the two tubes were then transferred as fast as possible, one after another, to two agarose Petri dishes (which had been pre-warmed as described earlier). After each dispensing the plate was tilted at every possible angle so as to ensure an even layer. A few moments of manipulation

should be enough, and the plates were allowed to stand on a pre-leveled solid surface at room temperature to allow permanent setting. The same was to be done to the rest of the plates. After ten minutes of standing of the last plate, all of the plates were incubated at 37°C for one hour, in an inverted position (in a moist incubator). Two ml of ten-fold diluted fresh GP or rabbit serum were then added to each plate. These were re-introduced into the incubation chamber and incubated further for 45 minutes. After this, the number of plaques appearing on the red cell lawn was counted with unaided eyes, or in doubtful cases, with the aid of a microscope (10X).

In the mouse anti-SRBC system, the concentration of RBC used was 25%, and the composition of the top layer is as follows: 0.4 ml of 0.83% agarose in distilled water, 0.4 ml 2-fold concentrated HEPES-Hanks', 0.1 ml SRBC and 0.1 ml of spleen cells. Fresh GP serum was used as a source of C. Incubation procedures were identical to that described above unless otherwise stated.

The relative compositions of the top layer in experiments using mastocytoma cells as indicator cells were the same in the mouse allogeneic system. The incubation procedure, however, was somewhat different. After the incubation with RC, the plates were washed once with HEPES-Hanks' and then 1.5 ml of HEPES-Hanks' supplemented with

10% fetal calf serum, 100 units penicillin and 100 µg streptomycin per ml was added to each plate. The plates were rinsed once with HEPES-Hanks', and then stained with Giemsa stain diluted 1:20 with physiological saline after fixation with 95% ethyl alcohol to facilitate plaque observation.

(d) Detection of Developed (7S) Plaques Using Developing Antiserum: The method by Sterzl and Riha (1965) and Dresser and Wortis (1965) was used. After the first incubation, 1.5 ml of prediluted rabbit anti-mouse gamma G antiserum was added onto the plates. They were incubated at 37°C for 1 hour. The serum was then aspirated off and the plates were washed once with physiological saline. Complement was then added and the plates further incubated at 37°C for 45 minutes. Plaques were counted after this incubation.

Inhibition of Plaque Formation: In experiments using alloantigens to inhibit plaque formation, the technique was identical to that described for the detection of direct plaques (see page 59), except that the composition of the top layer was as follows: 0.3 ml 0.83% agarose in double distilled water, 0.3 ml 2-fold concentrated HEPES-Hanks', 0.1 ml of antigen, 0.05 ml of spleen cells and 0.05 ml of 25% RBC in HEPES-Hanks'.

RESULTS

This chapter is divided into three sections: (A) studies for the development of a sensitive hemolytic plaque technique to detect antibody producing cells in transplantation immunity, (B) the investigation of the classes of antibodies involved in this assay, and (C) the study of the specificity of the plaque method.

(A) Attempts to Increase the Sensitivity of the Plaque Method

(a) Physical Variables: It was reasoned, a priori, that the number of indicator RBC as well as the total volume of the top layer would affect both the visibility of the plaques and the sensitivity of the method. For this reason, it was attempted to reduce the concentration of RBC and the volume of the top layer of agarose as far as possible, to obtain higher sensitivity without sacrificing the contrast essential for an unequivocal detection of plaques.

In determining the volume of the top layer, the relative amounts of the ingredients, viz., agarose in HEPES-Hanks', RBC and spleen cells had to be considered. Whenever too small a volume was poured onto the pre-warmed bottom layer (as described in Materials and Methods), this resulted in the formation of an uneven top layer, with clumps of RBC embedded within it. On the other hand a large volume resulted in a thick top layer which not only masked some of the plaques, but which also meant unnecessary waste of biological materials. After several attempts, it was concluded that the following composition of the top agarose layer was suitable for a high sensitivity and a good contrast: 0.3 ml of 2-fold concentrated HEPES-Hanks', 0.3 ml of 0.83% agarose in double distilled water, 0.1 ml of 12.5% RBC in HEPES-Hanks' and 0.1 ml of spleen cell suspension. In this way a thickness of

0.42 mm of the top layer was obtained*, compared with 0.88 mm and 0.89 mm in the methods of Jerne and Nordin (1963) and Hildemann and Pinkerton (1966), respectively.

(b) Choice of Sera as C Source: Rabbit and guinea pig sera are the most commonly used sources of C. Guinea pig serum was used by Gorer and O'Gorman (1956) and later by several authors for cytotoxic tests with alloantisera. However, Winn (1965) and Haughton and McGehee (1969) studied the H-2 allogeneic system by using rabbit C in the place of the usual GPC, and observed a higher cytolytic efficiency of mouse alloantibody. Therefore it was decided to compare the effectiveness of RC and GPC in revealing hemolytic plaques, using both xenogeneic (mouse anti-SRBC) and allogeneic (mouse) systems. Two groups of C57BL/6 mice were immunized with A strain SaI cells i.p. Three weeks later, they were given a boost of 4×10^7 A spleen cells i.p. Spleen cells of these mice were assayed for PFC 4 days after the secondary immunization using A strain RBC as target cells. Another group of C57BL/6 mice was given a single i.p. injection of 0.2 ml of 25% SRBC and the spleen cells assayed for PFC with SRBC 4 days later. Every Petri dish contained identical relative volumes of biological

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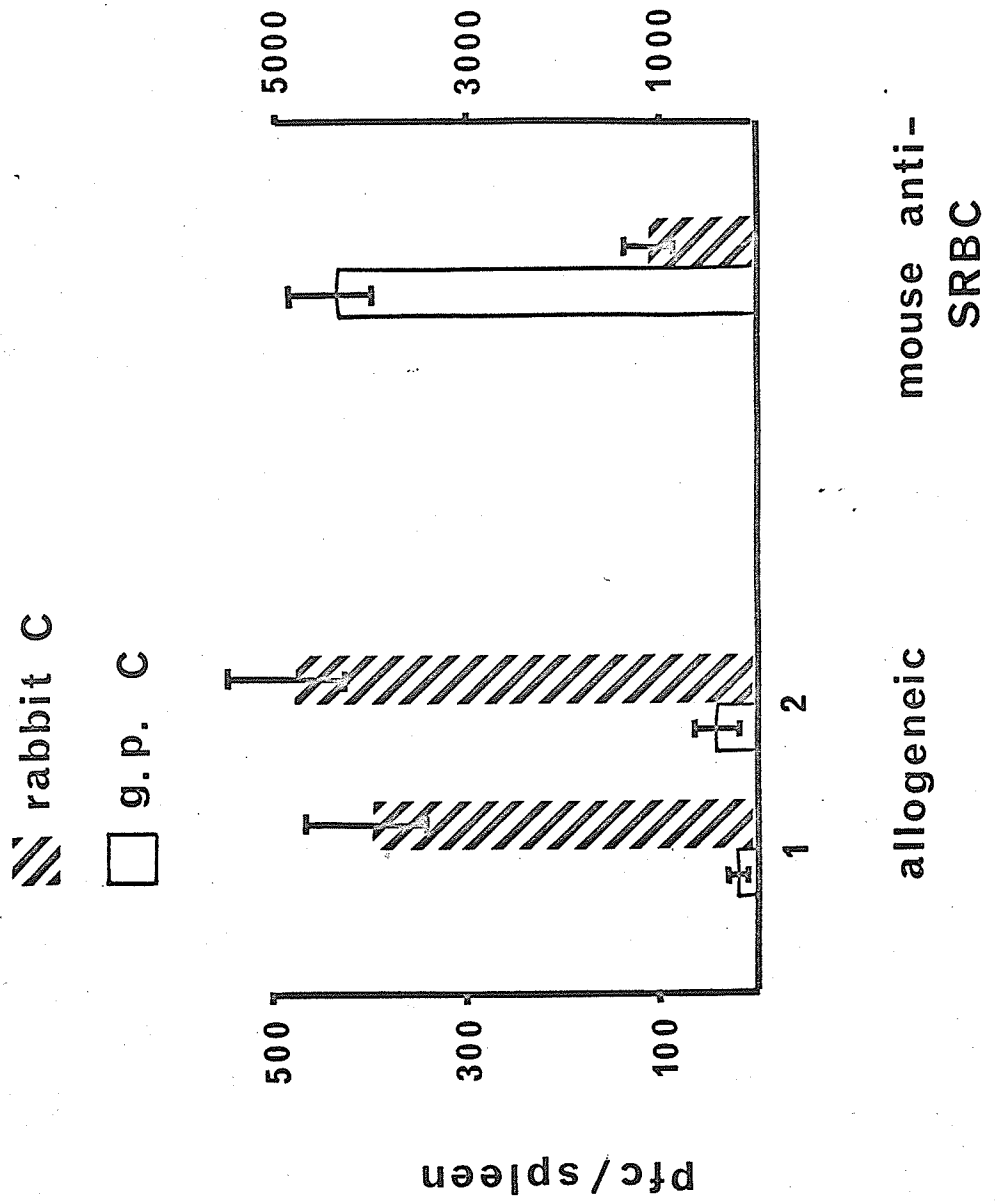
* Calculated with the formula: $\text{Thickness} = \frac{\text{Volume of top layer}}{\text{Flat area of Petri dish}}$

FIGURE 1. Comparison of the efficiency of rabbit complement and of Guinea pig complement in revealing plaques in the allogeneic (C57BL/6 anti-A) and xenogeneic (C57BL/6 anti-SRBC) systems. 1 and 2 refer to two separate experiments for the allogeneic system. Results are arithmetic means of triplicates. Cells from a pool of at least three spleens were used in each experiment.


rabbit C
g.p. C
5000
3000
1000
500
300
100
pfc/spleen
1
2
allogeneic
mouse anti-SRBC

materials to ensure a fair comparison. It can be seen from results reported in FIGURE 1 that RC was definitely more efficient than GPC in the allogeneic system, while the reverse was true in the mouse anti-SRBC system. The relative plaquing efficiency* for RC as compared with GPC is 16, i.e., RC could reveal 16 times more plaques than GPC in the allogeneic system while the ratio in the mouse-anti-SRBC system is 1/4. It was on the basis of these findings that RC was used in subsequent experiments in the allogeneic system.

(c) Attempts to Use an Anti-MRBC Antiserum (RAMRBC) as A Synergistic Antiserum: The following series of experiments was performed on the assumption that some cells producing antibody to red cell alloantigens may not result in plaque formation because either the antibodies have too low a hemolytic efficiency, or the plaque forming cell produces too small an amount of antibody. In order to reveal these hypothetical PFC of low hemolytic efficiency, it was considered possible that a xenogeneic antiserum added at sublytic concentrations might act synergistically with the alloantibody. In other words, two different antibody populations, one of allogeneic, and the other, of xenogeneic origin, each in concentration too low to induce hemolysis if present

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*Calculated by dividing the number of plaques obtained with rabbit serum as C source by that obtained with guinea pig serum as C source.

alone, might act synergistically to induce lysis of the target MRBC. And indeed, according to Ferrone et al., this synergistic action is the mechanism by which rabbit serum results to be more efficient than GP serum as a source of C in cytotoxic tests for human alloantibodies (Ferrone et al., 1971). For this purpose a rabbit anti-MRBC antiserum was produced by four injections of C57BL/6 RBC into a young adult rabbit, and the serum was collected 10 days after the last injection as described in Materials and Methods. To determine the synergistic effect with alloantibodies, this RAMRBC serum should be used at a sub-hemolytic concentration. Such a sub-hemolytic concentration was determined with the following experiment.

A series of Petri dishes was prepared with a top layer of strain A MRBC at the same concentration as used in the plaque assay, and spleen cells were deliberately included (as they would be there in the future plaque assays). After the top layer had solidified at room temperature, 2 ml of fresh rabbit serum (dilution 1/10) as a source of C together with the RAMRBC serially diluted in HEPES-Hanks' without phenol red were added onto the dishes. These were incubated at 37°C for one hour. The supernatant was then taken out and the O.D. was read at 550 mμ to measure the amount of hemoglobin released in the medium. As control the RC was heat inactivated at 56°C for 30 min. before

OPTICAL DENSITY (O.D.) AT 550 mμ

	Amount RAMRBC Per Plate (ml)		
Dilution	0.1	0.2	0.4
1/1	N.R.*	N.R.	N.R.
1/10	0.435	0.495	0.581
1/100	0.334	0.394	0.485
1/1000	0.286	0.358	0.402
1/2000	0.278	0.270	0.303

Control (RC heated at 56°C for 30 min. before mixing with 0.1 ml of 1/10 dilution of RAMRBC): 0.268

TABLE I. Hemolytic activity (O.D. at 550 mμ) of RAMRBC at different volume/dilution combinations. Results are arithmetic means of quadruplicates in a single experiment.

* At this high concentration of RAMRBC the supernatant became fairly turbid, giving unusually high O.D. readings.

mixing with 0.1 ml of 1/10 dilution of RAMRBC. The results of such an experiment are reported in TABLE I. It can be seen that at a dilution of 1/2000, the RAMRBC showed no hemolytic activity, i.e., the O.D. stayed at more or less the control value regardless of the volume of RAMRBC used.

The capacity of the RAMRBC serum to affect the number of plaques in the C57BL/6 anti-A system was then studied. C57BL/6 mice were primed with SaI cells as described in Materials and Methods. After three weeks these animals were given a secondary immunization of an intraperitoneal injection of 4×10^7 A spleen cells. PFC were then assayed four days later, following the experimental protocol outlined in FIGURE 2. It can be seen from the results reported in TABLE II that generalized hemolysis was obtained at dilutions lower than 1/1000, whilst at 1/1000 or higher (experiment 3(A)), RAMRBC tend to decrease rather than increase the number of plaques. This tendency was seen regardless of whether the antiserum was added at the same time as (A) or half an hour before (B) the addition of RC. It was concluded from these results that a rabbit anti-MRBC antiserum does not increase the sensitivity of detection of PFC and may actually decrease it.

In spite of the fact that there was no indication that

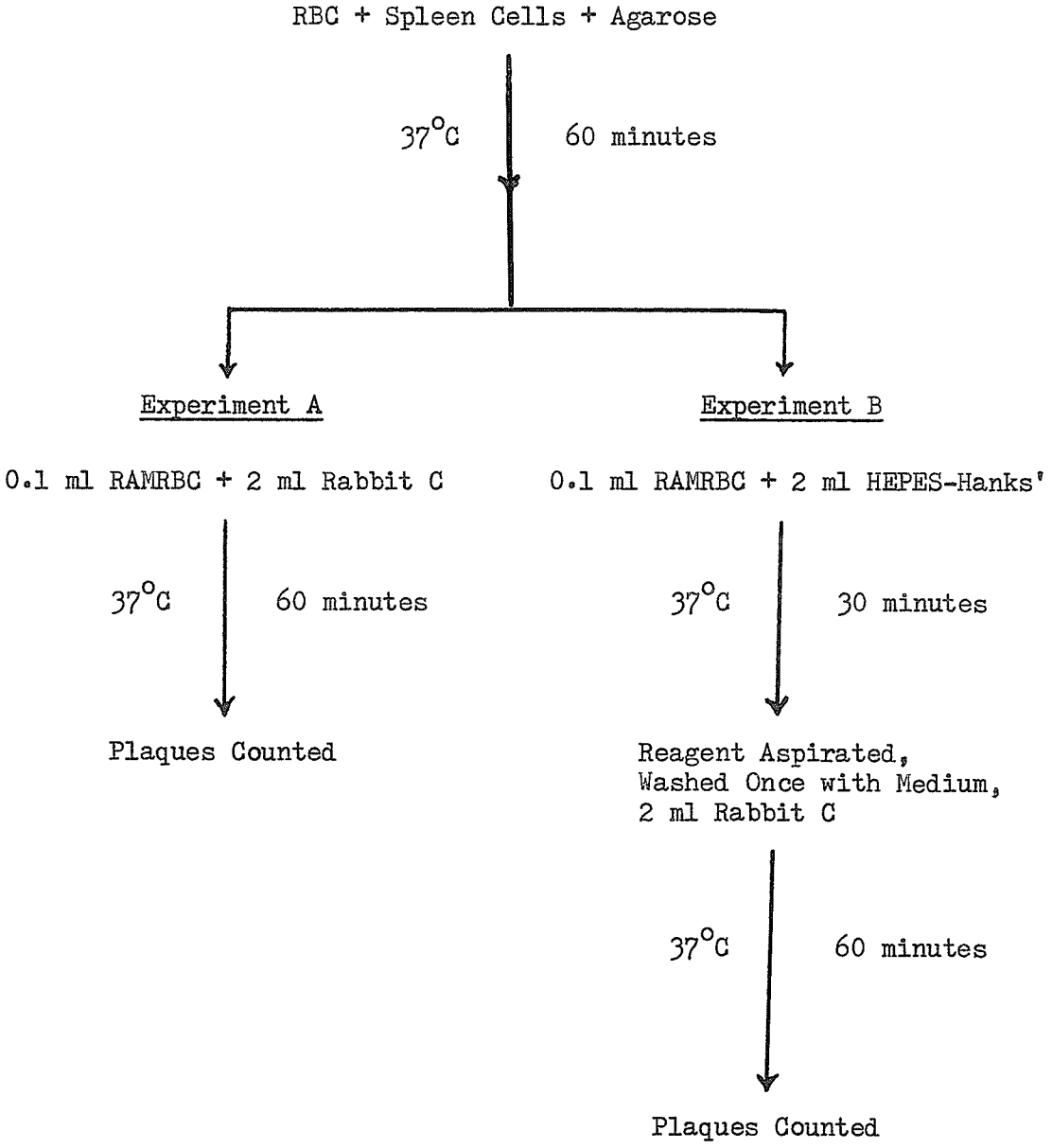


FIGURE 2. Flow diagram of the plaque method employing RAMRBC serum in an attempt to increase the sensitivity of the technique.

PLAQUES PER PLATE ($2X10^7$ spleen cells)

Experiment	DILUTIONS OF RAMRBC					
	1/10	1/100	1/1000	1/2000	1/4000	No RAMRBC added
1 (A)	H	47(44-52)	64(60-67)	N.D.	N.D.	65(58-67)
(B)	H	H	48(45-52)	N.D.	N.D.	67(60-69)
2 (A)	H	H	20(18-22)	N.D.	N.D.	34(25-38)
3 (A)	N.D.	N.D.	79(71-88)	75(70-80)	75(72-79)	78(74-83)

TABLE II. The effect of the inclusion of RAMRBC serum on the number of plaques formed by immune C57BL/6 spleen cells on a layer of A RBC. The results are arithmetic means of triplicates with the ranges in parentheses. A and B are two versions of the same experiment as described in FIGURE 2. RC was used.

H: Generalized hemolysis.

N.D.: Not done.

natural anti-mouse antibody, which is present in every rabbit serum (Boyse et al., 1970), may also interfere with the detection of PFC, this was considered possible, and therefore, it was decided to use a rabbit serum freed, as far as possible, of natural anti-mouse antibodies as the source of C. Absorption of natural antibodies may be achieved with the use of mouse cells (Boyse et al., 1970). However, this would result in the loss of C activity unless C fixation is prevented by performing the absorption procedure in cold temperature (see Mayer, 1965) or in the presence of an excess of EDTA which removes any divalent ions thus preventing C fixation (Boyse et al., 1970).

In view of the large volume of fresh serum used as the source of C in the plaque assay, using mouse cells as the natural antibody absorbent would be impractical and very expensive. Therefore, a more economical method was sought. Cohen and Schlesinger (1970) reported their successful attempt to absorb natural anti-mouse antibodies from fresh normal serum with the relatively inexpensive agarose. The effectiveness of this absorption method was tested by measuring the cytotoxic effect of the absorbed RS on thymus cells, while the complement activity of the absorbed RS was tested with the use of thymus cells sensitized with an allo-antiserum. For this purpose strain A thymus cells were labeled with ^{51}Cr as described elsewhere and divided in two

portions, one of which was left unincubated, and the other was incubated with a C57BL/6 anti-A alloantiserum at a dilution of 1/500, which was known to be capable of sensitizing the specific target cells to such an extent that 100% lysis would occur in the presence of excess C (see FIGURE 14), thus releasing radioactive Cr in the supernatant.

The absorption procedure was as described in Materials and Methods. Briefly, fresh rabbit serum was mixed with agarose (SeaKem) in the ratio of 3 ml to 0.08 gm in the presence of EDTA. Absorption was allowed to take place for 30 minutes with constant mixing of the suspension kept in an ice bucket. After reconstituting its Ca^{++} content with $CaCl_2$, the serum was used in the cytotoxic test. For control purposes, the unabsorbed RS was also treated with EDTA and reconstituted with $CaCl_2$ but was not absorbed with agarose.

Fresh rabbit serum whose natural antibody content was to be tested was serially diluted in 0.1 ml volumes in the wells of two MicroTest II plates. To the wells of one plate were added 1×10^5 sensitized cells contained in 0.15 ml. After sealing the plate with a plate sealer, it was incubated at $37^{\circ}C$ for 30 minutes. The supernatants in the wells (after spinning the plate at 350 g for 4 minutes) were taken out for the measurement of radioactivity. This measures the C level in the test serum. To the other plate, 1×10^5 unsensitized

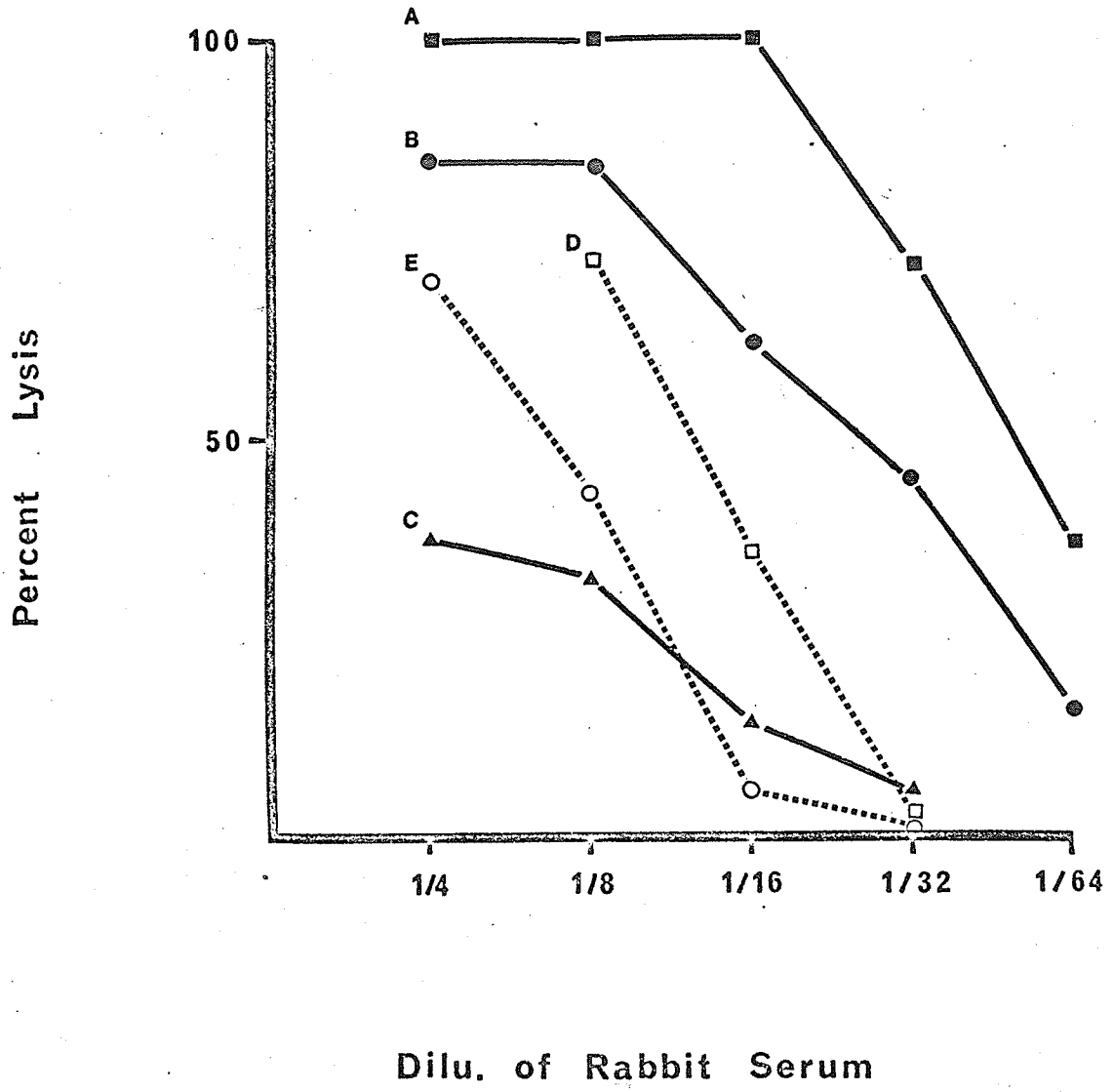
cells contained in 0.1 ml were added. Incubation was carried out at room temperature for 15 minutes. As a source of C, 0.05 ml of 1/6 diluted fresh rabbit serum, which was absorbed twice with agarose, was added to the wells. The plate was incubated further for 30 minutes at 37°C. The rest of the procedure was the same as described above. This procedure measures the natural antibody level in the test serum. The results are shown in FIGURE 3.

A comparison of the curves E and D demonstrates that the absorption procedure resulted in a reduction, though not a complete elimination of the natural antibody activity of the RS, while a comparison of the curves B and C demonstrates that the use of EDTA effectively reduced the undesirable loss of C activity during the absorption procedure. However, a slight loss of such activity, even in the presence of EDTA, is suggested by the comparison of curves A and B. It was concluded that this method of absorption is adequate for practical purposes to reduce the natural antibody levels of normal rabbit sera, and that, to increase its effectiveness, it would be desirable to use a serum with very low level of natural antibody to start with.

Using the same cytotoxic assay a total of fifteen rabbits were screened for the natural anti-mouse antibodies. All were found to contain natural antibodies at different titres. Two rabbits with

FIGURE 3. Absorption of natural anti-mouse antibodies from rabbit serum using agarose in the presence of EDTA. Each point represents the arithmetic mean of triplicates performed in a single experiment. Please see text for explanation of the experiment.

TARGET THYMUS CELLS		TREATMENTS OF C
SENSITIZED	A	UNABSORBED, EDTA
	B	ABSORBED, EDTA
	C	" , NO EDTA
NON-SENSITIZED	D	UNABSORBED, EDTA
	E	ABSORBED, EDTA



the lowest levels were chosen and kept for constant supply of fresh sera. Before each plaque experiment the rabbit serum was absorbed with the method described above. The effectiveness of absorbed RS as C source in the plaque method was then tested by comparing the C activity of absorbed and unabsorbed RS in inducing plaque formation. TABLE III shows that the absorbed RS gives slightly more plaques than the unabsorbed one. Although this difference is not statistically significant, this finding nevertheless confirms that the absorption of natural antibody from the RS does not reduce the effectiveness of this serum as the source of C in the plaque assay.

PLAQUES PER 2 X 10^7 SPLEEN CELLS

Experiment	Absorbed RS	Unabsorbed RS	% Increase
1	51 (46-59)	48 (45-50)	6.3
2	85 (70-105)	79 (68-85)	7.6

TABLE III. Comparison of usage of normal rabbit serum (RS) absorbed with agarose in the presence of EDTA, and RS without absorption as sources of C in the allogeneic plaque method. Results are arithmetic means of triplicates from a pool of three spleens. Ranges are in parenthesis. RC was used.

(d) Pretreatment of MRBC with Neuraminidase (VCN): There have been numerous reports concerning the action of neuraminidase (VCN) on cell membranes and the resultant increase of their susceptibility to alloantibodies. For example Sanford (1967) reported that A TA-3 tumor would not kill recipient mice if the cells were treated in vitro with neuraminidase before i.p. injection; Currie et al. (1968) showed that mouse trophoblast could be made to express histocompatibility antigens by the enzyme treatment, and Schlesinger and Amos (1971) reported that neuraminidase treatment increased the sensitivity of thymus cells to the cytotoxic effects of guinea pig serum, θ-C3H antibody and TL-allo-antigen antibody. The prevailing hypothesis for the action of this enzyme is that of 'unmasking' of antigens on the cell surface. Apffel and Peters (1967) suggested that masking of antigens is one of the normal natural biological roles of glycoproteins, and that sialoglycoprotein on the cell surface may be a distinct system of immunoregulation. Thus, by removing the sialic residues of glycoproteins on the cell surface with the neuraminidase treatment, antigenic determinants hitherto hidden would be exposed and react with the specific antibodies.

In view of these findings, it was considered likely that MRBC pretreated with the enzyme could be used to increase the sensitivity of the method of PFC detection. Pretreatment of MRBC was carried

out at 37°C for 30 minutes with different concentrations of VCN. After incubation the MRBC were washed three times with cold HEPES-Hanks', and the procedure for detection of PFC was performed as usual using MRBC incubated in absence of VCN and non-incubated as controls. The results in TABLE IV showed that VCN did not significantly increase the number of plaques, regardless of its concentration used to treat the RBC. It should be mentioned that the red blood cells after treatment had remained healthy both macroscopically, (showing no sign of spontaneous hemolysis) and microscopically (being morphologically normal).

NON-INCUBATED	VCN PRETREATMENT (units/10^9 RBC)		
	250	50	0
109(97-118)	103(95-116)	93(79-110)	96(88-102)

TABLE IV. Neuraminidase (VCN) pretreatment of MRBC at 37°C for 30 minutes before incorporation in the top layer. Units indicated are concentration of the enzyme present in a particular incubation lot. Results are arithmetic means of triplicates. Ranges are in parenthesis.

Discussion of Section (A) and Estimation of the Sensitivity of the Technique Developed: In summary a few variables were found to be important to increase the sensitivity of the technique to detect PFC against mouse alloantigens.

(1) It was found that the top layer could be considerably

reduced in volume without inducing any inconveniences. The advantages with the reduction of the top layer were:

i) clearer plaques because of the thinness of the layer,

ii) the need of a smaller amount of biological materials with

iii) a probable increase of sensitivity because less antibody was required to form a plaque.

(2) The choice of C is important. RC was found to be very efficient in the present system, although it was excelled by GPC in the mouse anti-SRBC system. This finding is in line with the observation by Haughton and McGehee (1969) who found that serum cytotoxic antibodies killed allogeneic lymph node cells more readily in the presence of RC than GPC. Other similar findings were reported by Boyse et al. (1962) and Winn (1965).

(3) Although successful in studies by other authors in other systems (Winn, 1965; Moller, G., 1963; Nelken et al., 1970), the addition of a rabbit antiserum to MRBC in an attempt to increase the sensitivity of the latter

to hemolytic antibodies seemed to induce inhibition of plaque formation, rather than acting as a synergistic agent. Since such rabbit antiserum would be expected to favor hemolysis whenever other hemolytic antibodies are present on the target MRBC in a concentration too low for lysis, the failure to obtain any synergistic effect may indicate that no "sub-threshold" PFC were present, or in other words, that the sensitivity of the method was already maximal. The finding that VCN did not increase the plaque number further supports this hypothesis. Obviously these considerations do not apply to cells producing antibodies which are non-hemolytic. The failure to obtain a synergistic effect indicated also that it might be desirable to use as a source of C a serum with as little as possible natural anti-mouse antibodies. For this reason the rabbit serum donor (for RC) with the lowest natural anti-mouse antibody level was chosen from among fifteen animals and then further absorbed to reduce natural antibodies.

The technique which was finally adopted was compared with other methods to establish its relative sensitivity. No direct

comparison was made with the method described by Hildemann and Pinkerton (1966), since this method was found to be not easily reproducible. However, the present technique was able to detect 115 PFC per 1 X 10^7 spleen cells at the peak response (see FIGURE 10), in comparison with 26.6 PFC in a comparable study as reported by Hildemann and Pinkerton (1966).

The method reported here was also compared with that of Nordin, Cerottini and Brunner (1971) which requires the use of tumor cells (Mastocytoma P-815-X2, an ascitic tumor indigenous to DBA/2 (H-2^d)) as targets to detect PFC. The procedure of the technique of these authors was followed as close as possible, except that the experiment was performed with plastic Petri dishes (Falcon), instead of the microscopic slides (75 x 50 mm) used by these authors. TABLE V shows the comparison of the number of direct plaques detected by using the two different target cells: DBA/2 RBC and mastocytoma P-815-X2 (indigenous to DBA/2). It can be seen that the number of plaques detected with mastocytoma as target was only about 10% of that detected with RBC as target. Since the number of PFC detected in this experiment is of the same order of magnitude as that reported by Nordin et al. (1971), it can be concluded that the hemolytic plaque method is far more sensitive than its counterpart. It should be noted also

that the method requiring tumor cells as targets calls for a cumbersome incubation procedure (see Materials and Methods). Moreover it was also observed that the plaques on the tumor cell lawn were less distinct (fuzzy appearance) than the hemolytic plaques, presumably due to the much larger sizes of the target tumor cells.

Experiment	Plaques per 2 x 10^7 Spleen Cells	
	Target Cells	
	Mastocytoma (P-815-X2)	DBA/2 RBC
1	5.0 (4-6)	63 (58-66)
2	5.3 (5-6)	42 (36-52)

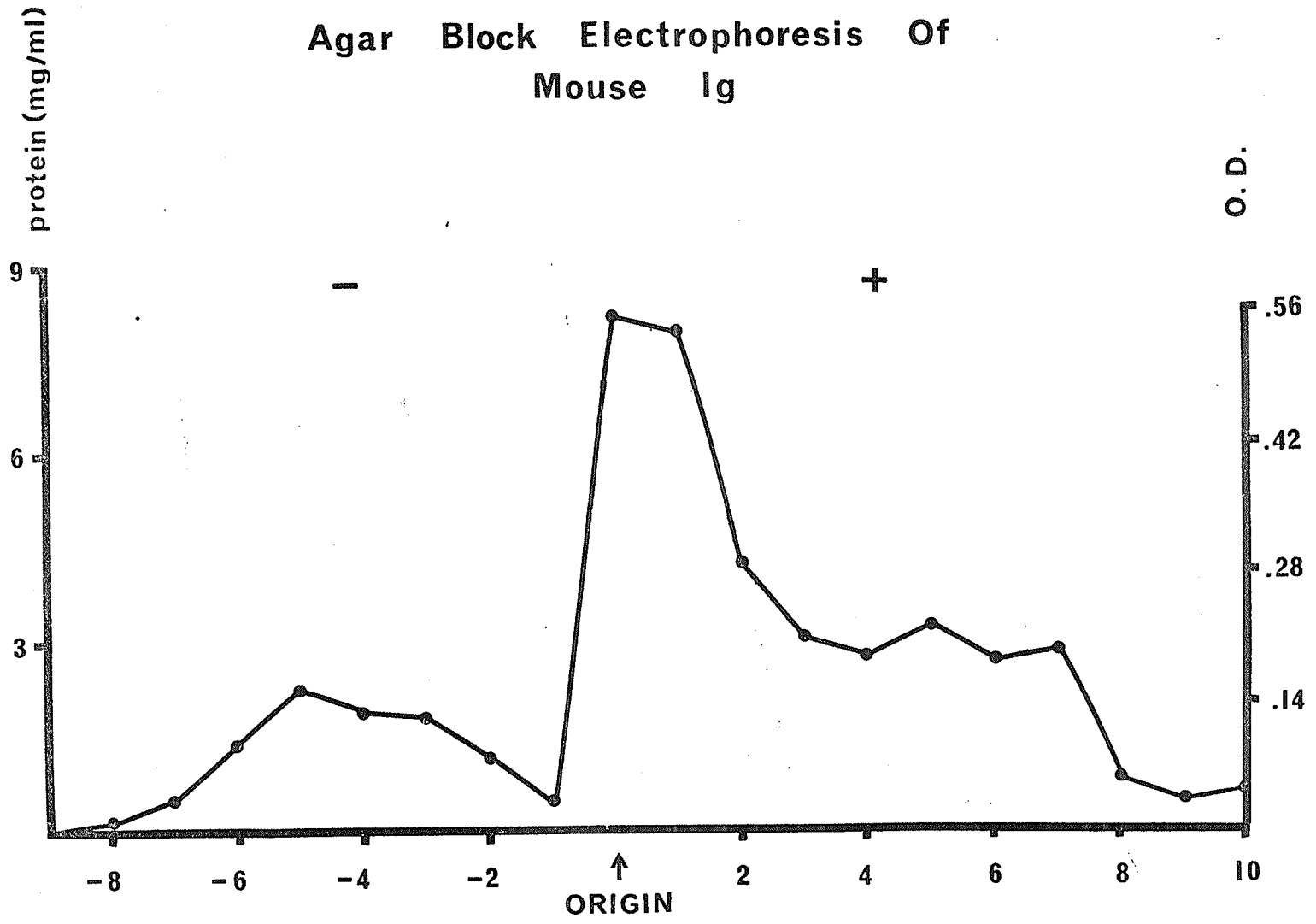
TABLE V. Comparison of using mastocytoma (P-815-X2) and MRBC, both of the donor allele ($H\text{-}2^d$), as target cells for detection of PFC from spleens of C3H ($H\text{-}2^k$) mice immunized once with 3 x 10^7 mastocytoma cells 6-7 days earlier (experiments 1 and 2 respectively). Results are arithmetic means of triplicates from a pool of four spleens. Ranges are in parentheses. RC was used.

(B) Study of the Antibody Classes Involved in Plaque Formation

In transplantation immunity, it is known that both IgM and IgG alloantibodies may be produced (Moller, G., 1966). However, very little information is available as to the kinetics of appearance of cells producing IgM and IgG, respectively. Moreover, it was essential to study the class of antibodies produced by the PFC detected with the present method, since rabbit C may produce hemolysis with antibody classes which are not hemolytic if tested with GPC.

It is well known that the original method for the detection of PFC developed by Jerne and Nordin (1963), which employed C alone, detects only PFC of the IgM class, or direct plaques. IgG plaques are not seen in this case. In 1965, Dresser and Wortis, and Sterzl and Riha independently reported the use of a xenogeneic anti-mouse gamma G antiserum to detect IgG plaques (indirect or developed plaques). This phenomenon can be attributed to the fact, as discussed by Humphrey and Dourmashkin (1965), that one IgM molecule alone can produce a functional lesion on the cell membrane, while two adjacent IgG molecules on the cell surface are required to produce the same result. The addition of anti-gamma G antibodies increases the likelihood that an IgG molecule be accompanied by an adjacent antibody molecule to fulfill its cytolytic function (see also discussion on page 32).

FIGURE 4. Agar block electrophoresis of mouse immunoglobulin. Protein concentration of eluates from agar blocks after electrophoresis were determined using Beckman 1098 spectrophotometer at 280 mμ. - and + represent the cathode and anode ends, respectively. Algebraic numbers on the abscissa refer to the agar strips relative to the origin.


Agar Block Electrophoresis Of
Mouse Ig
protein (mg/ml)
O. D.
9
6
3
.56
.42
.28
.14
−
+
−8
−6
−4
−2
ORIGIN
2
4
6
8
10

For these reasons, it was decided to study the nature of the antibodies released by the PFC with two methods: i) the use of a rabbit anti-mouse gamma G as a developing antiserum (DA), and ii) the use of concanavalin A (con A) which has been claimed to be capable of distinguishing 19S from 7S plaques (Nordin et al., 1969).

(a) Production and Specificity of the Rabbit Anti-Mouse Gamma G Antiserum: Mouse gamma globulin was prepared from 40 ml of heat-inactivated normal A serum by 50% $(NH_4)_2SO_4$ precipitation. The crude preparation was then partially purified by agar block electrophoresis, as described in Materials and Methods. After electrophoresis, the agar block was cut into strips of 8 x 1 cm each, which were then individually introduced into 15-ml test tubes. These strips as contained in the test tubes were submitted to freezing and thawing three times so as to release the serum proteins contained in them. The protein content of these fractions was then determined by reading the optical density at 280 mμ (Beckman 1098). The protein distribution in relation to the direction of electrophoresis is shown in FIGURE 4. In order to determine the corresponding distribution of gamma G protein in the agar strips, analytical immunodiffusion was done in the following manner. Agar slides (7.5 x 5.0 cm) were prepared with 1% agar in borate buffer of 0.005M, pH 8.0. As many holes, each of

diameter of about 1.5 mm, as there were agar strips were punched along the major axis on the agar slides. Into each hole was introduced the respective eluates from the strips. A rabbit anti-mouse gamma G_2 antiserum and a rabbit anti-mouse whole serum (MWS) were used as the antibody reagents. The slides were incubated at room temperature for 24 hours in a moist chamber. The distribution of mouse gamma G_2 is indicated in FIGURE 5. It can be seen that there are virtually no precipitin lines with anti-mouse gamma G_2 serum on the anode side. On the other hand, a precipitin line is observed on the cathode side with this serum. From strip -5 to -8 only a single line is observed with both anti-mouse gamma G_2 and anti-MWS. An anti-mouse gamma M antiserum was used to check the possible contamination of the fractions of the cathode side with this other major immunoglobulin class (lower slide of figure). It can be seen that no precipitin lines are present, indicating the absence of gamma M immunoglobulins in these agar strips proven to contain gamma G_2. Eluates from -5 through -8 were then pooled and concentrated by dialyzing against borate buffer, 0.005M, pH 8.0, under negative pressure. Protein concentration of the pooled concentrated fraction was then determined by the Lowry method (Lowry et al., 1951).

The purity of this concentrate was further checked by

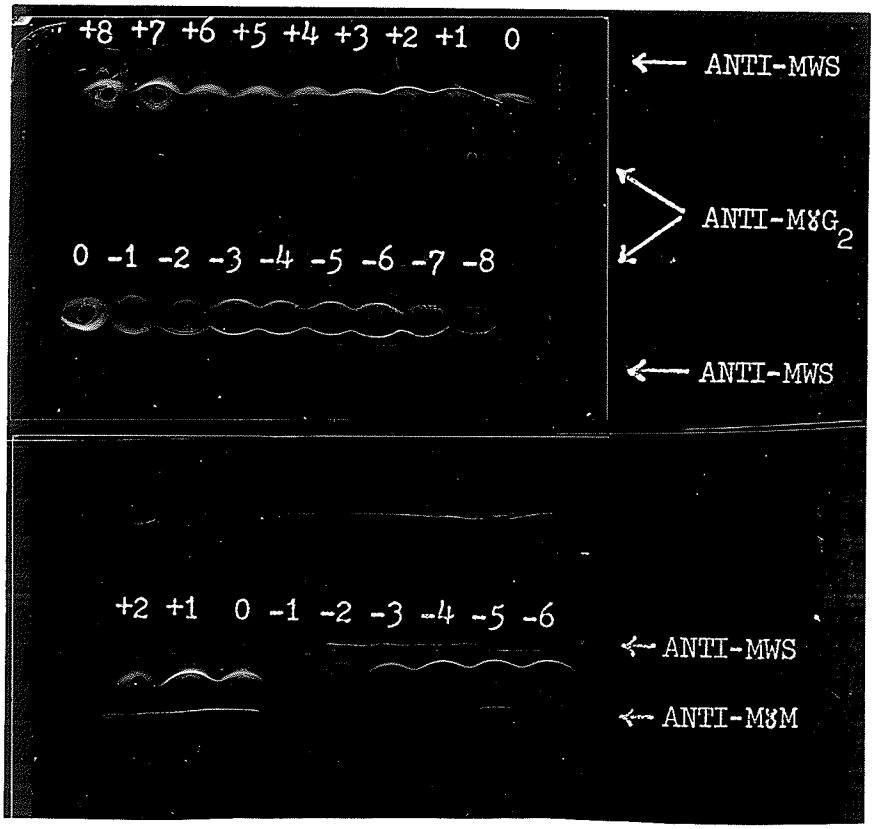


FIGURE 5. Analytical (double) immunodiffusion for the determination of the location of mouse gamma G_2 immunoglobulins after agar block electrophoresis. Each hole in the agar slides contains the eluate from one strip (1x8 cm). Algebraic numbers refer to the positions of the samples relative to the origin. The upper slide represents a test for gamma G_2, while the lower one represents a test for gamma M.

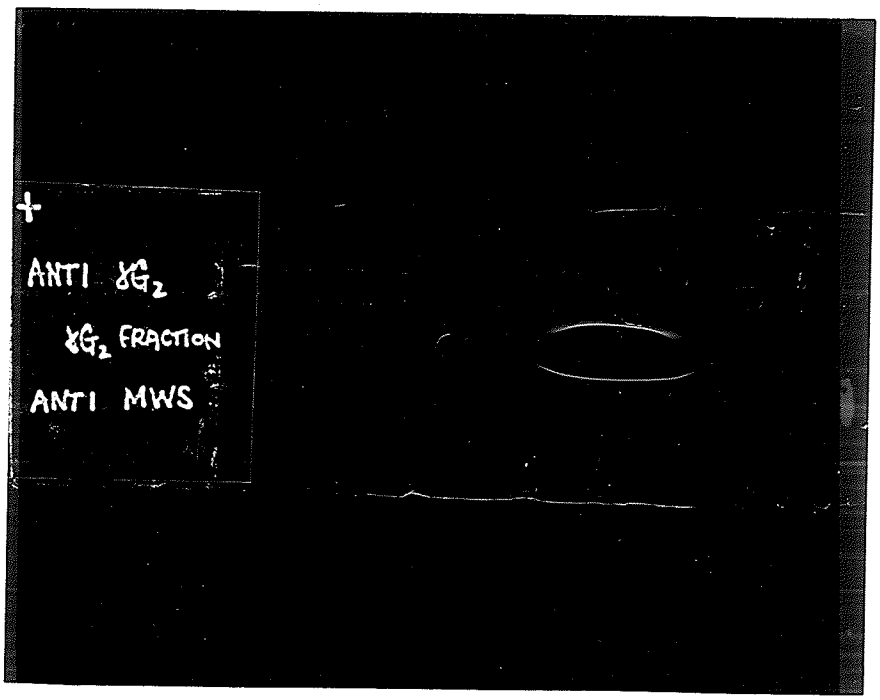


FIGURE 6. Immunoelectrophoresis of the concentrated gamma G_2 fraction. Anti-mouse gamma G_2 and anti-mouse whole serum (MWS) were used as the antibody reagents.

immunoelectrophoresis, again using the above-mentioned anti-MWS and anti-mouse gamma G_2 antisera as the antibody reagents. The result in FIGURE 6 shows that the preparation was reasonably pure since one single line was obtained with the anti-mouse gamma G_2, and an additional very faint, closely associated one with the anti-MWS, indicating the absence of contamination by immunoglobulin classes other than gamma G. The purity and the composition in terms of gamma G subclasses of this gamma G preparation was not further checked since for the purpose of this investigation an antiserum to immunoglobulins of all IgG subclasses was desirable. This preparation was then used as the immunogen for the production of rabbit anti-mouse gamma G antiserum following the procedure described elsewhere.

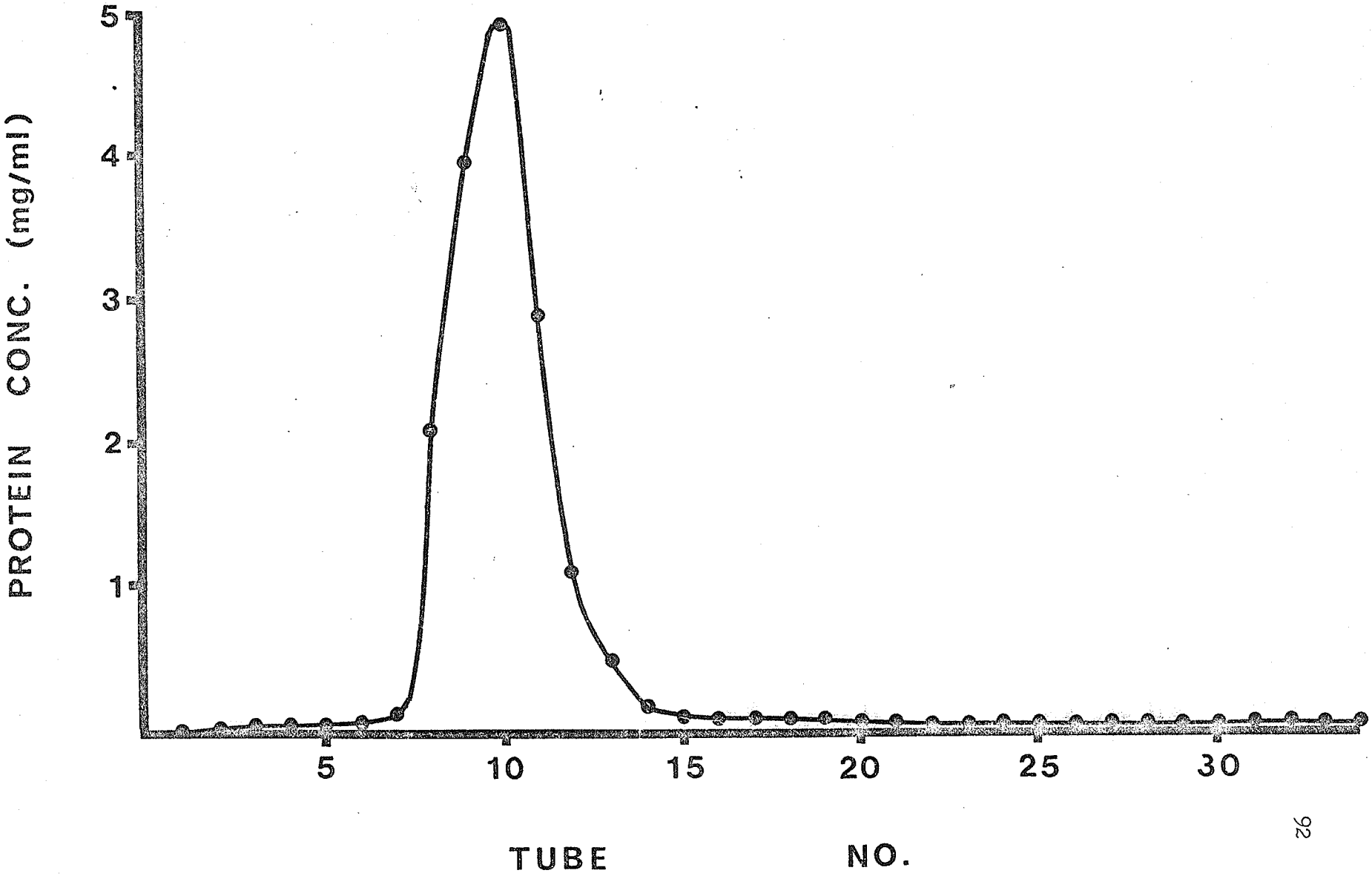
However, to obtain an antiserum specific for mouse gamma G, the use of a purified antigen is not sufficient. It is known that mouse IgM and IgG molecules possess the same kappa light chains (Potter, 1967). Therefore, the rabbit anti-gamma G serum was expected to react with IgM molecules as well (thus interfering with IgM plaque formation (Nossal et al., 1971)). In order to render the antiserum specific for gamma G only, the anti-K light chain antibodies had to be absorbed out. For this reason a K-type Bence Jones protein (BJP) was prepared as described in Materials and Methods. After dialysis against

0.05M Tris-acetate buffer, pH 5.5, the concentrated urine sample was passed through a DEAE-cellulose column equilibrated with the same buffer. The eluate was collected in 6-ml fractions. Optical density of the fractions was read, and the protein concentration was calculated using extinction coefficient $E^{1\%}_{1\ cm}{}_{280\ mu} = 11.4$. The protein profile is shown in FIGURE 7. A clear-cut single peak was obtained. Consequently tubes 6-17 were pooled and concentrated as described elsewhere.

The purity of this KBJP concentrate was checked with immunoelectrophoresis, using a non-purified anti-KBJP antiserum (gift from Dr. Tsay, Department of Immunology, University of Manitoba) and anti-mouse whole serum antiserum as the antibody reagents. It can be seen from FIGURE 8 that, with either of these, the KBJP concentrate formed a single precipitin line, indicating that the sample was reasonably pure.

The optimal amount of BJP for absorption was determined by the two-drop test (see page 55). It was found that the tube with maximal precipitate was the fourth tube of the series of two-fold dilutions corresponding to a BJP concentration of 15 μg N/ml. Consequently an amount of X ml of the anti-mouse gamma G antiserum would require 15X μg N of BJP for absorption. The absorption procedure was carried out as described elsewhere. The absorbed antiserum will be

FIGURE 7. DEAE-cellulose (0.05M Tris-acetate buffer, pH 5.5) elution pattern of K-type Bence Jones protein from the urine of MOPC-149 myeloma bearing BALB/c. Fractions are in 6 ml portions.


PROTEIN CONC. (mg/ml)
5
4
3
2
1
5
10
15
20
25
30
TUBE NO.

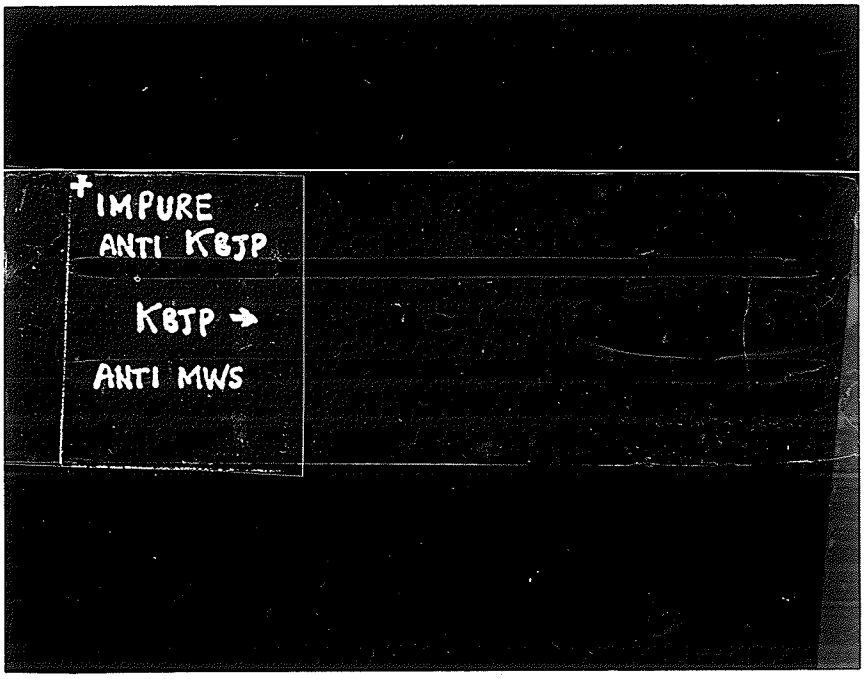


FIGURE 8. Immunoelectrophoresis of Kappa-type Bence Jones protein (KBJP) concentrate. The purity of the preparation was checked with a non-purified anti-KBJP antiserum and an anti-mouse whole serum (MWS) antiserum.

referred to as DA (developing antiserum). This DA was not further checked for specificity. However, a two-drop test indicated that the absorption procedure used in this study was adequate, since no precipitate was observed upon mixing this DA and BJP for the second time.

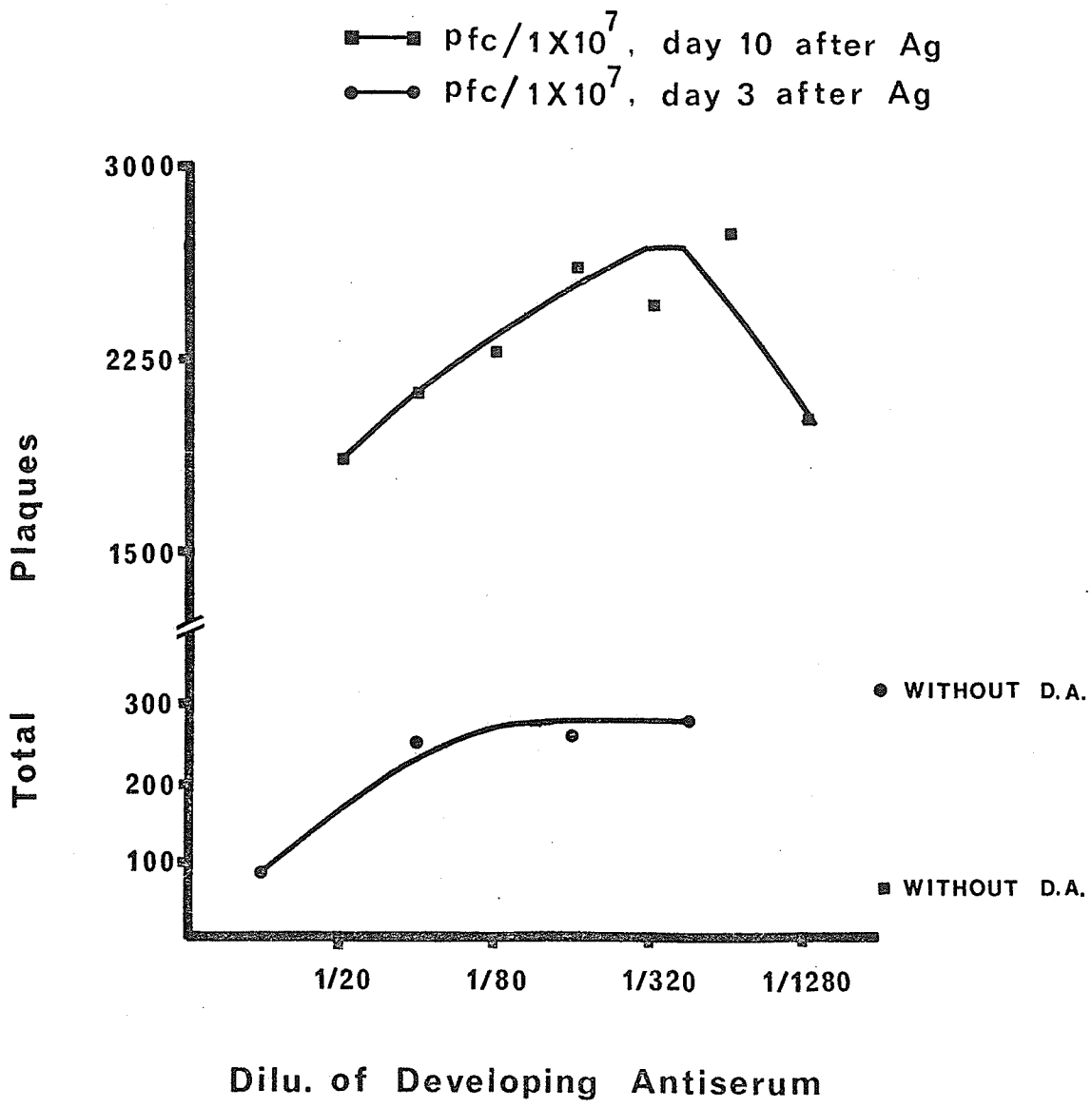
It is well known that developing antisera specific for the gamma G subclasses could reveal plaques of the corresponding subclasses (Nossal et al., 1971). For example an antiserum specific for gamma G_1 would develop IgG_1 plaques; another specific for gamma G_{2a} would develop IgG_{2a} plaques, etc. Although the antibody reagent used to recognize the gamma G immunoglobulins in this study had been anti-gamma G_2 throughout the preparation procedure, a pure gamma G_2 preparation was probably not obtained because of the close association with the other gamma G subclass in the electrophoresis pattern (see Potter, 1967). Consequently the developed plaques obtained with DA should be classified under the general name of IgG plaques. This is acceptable, since in the present study, the interest lay in the developing of plaques of the IgG type in general.

(b) <u>The Use of the Developing Antiserum (DA) in the Xenogeneic and Allogeneic Plaque Systems</u>: The developing antiserum (DA) was first tested of its potential in developing 7S plaques by means of some preliminary experiments with the well established anti-SRBC system.

The experimental design was based on three well known facts: (1) a developing antiserum of this kind might interfere with 19S plaque formation if too concentrated (Sterzl and Riha, 1965; Chou et al., 1967). (2) there is an optimal dilution for maximal revelation of 7S plaques (Sterzl and Riha, 1965; Chou et al., 1967), and (3) 19S plaques predominate in the first 5-7 days, while 7S plaques gradually increase in number later (Sterzl and Riha, 1965). Theoretically there usually is a dilution at which 7S plaques could be maximally developed without significantly affecting 19S plaques.

With these facts in mind, the study was carried out with two experimental groups of C57BL/6 mice which had been immunized once with 0.2 ml of 25% SRBC intraperitoneally. One group was killed three days later (when 19S plaques are almost exclusively found), and another group, ten days later (when 7S plaques predominate). PFC from the spleens of these animals were studied (see Materials and Methods) with serial dilutions of the DA in order to establish the optimal dilutions for developing 7S plaques with a minimal inhibition of 19S plaques. FIGURE 9 shows the results of such a study. The number of plaques recorded is the total number appearing in a plate. It can be seen that the day 3 graph gives a marked inhibition at high concentrations of the DA and it begins to level off between the dilutions 1/40 and 1/160,

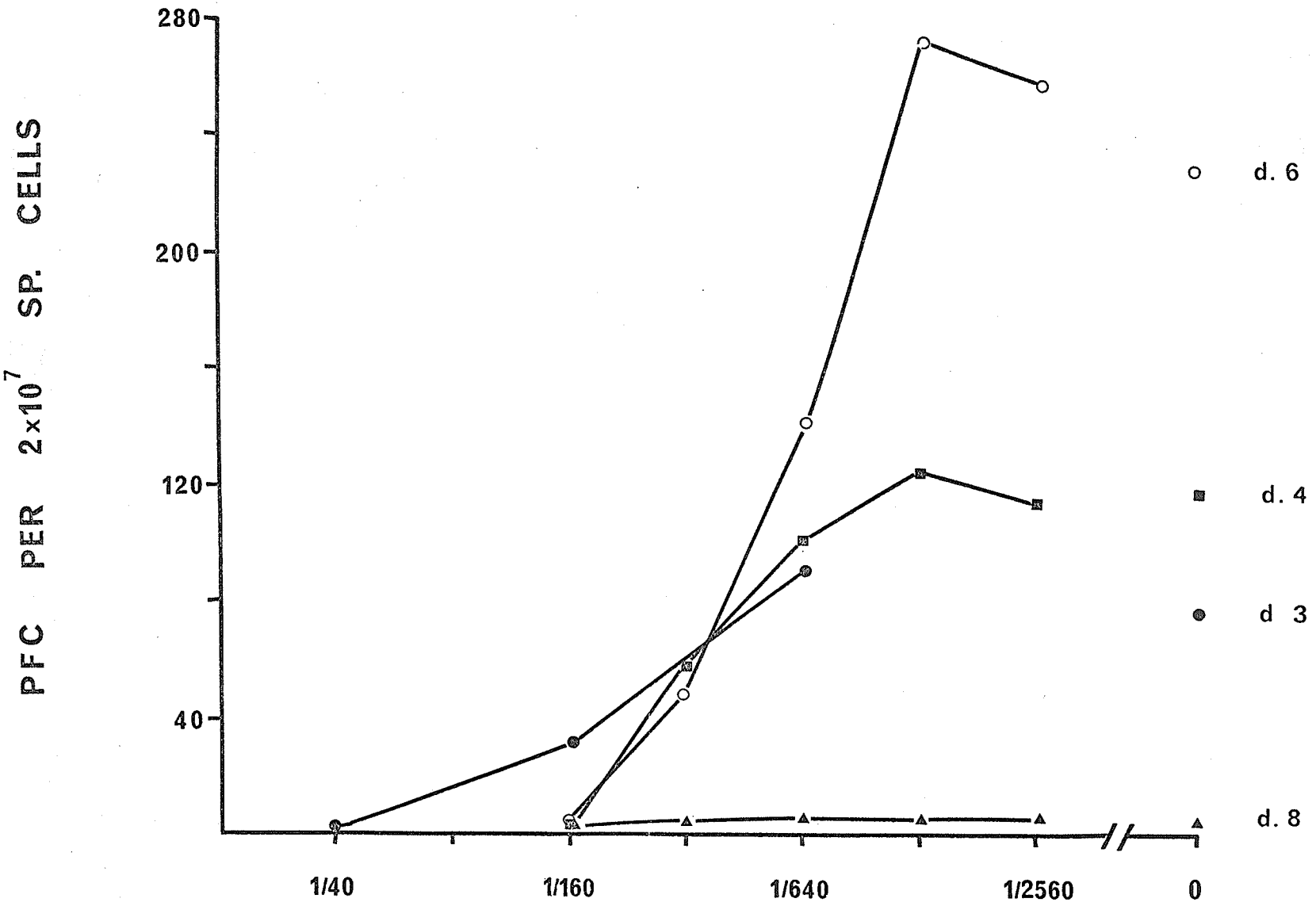
FIGURE 9. The effectiveness of DA in developing 7S plaques in the mouse anti-SRBC system. The two graphs represent experiments with spleen cells obtained on day 3 and day 10 after antigen injection. Each point represents an average of triplicates from a pool of four spleens. GPC was used.


pfc/1X10^7, day 10 after Ag
pfc/1X10^7, day 3 after Ag
Total Plaques
3000
2250
1500
300
200
100
WITHOUT D.A.
WITHOUT D.A.
1/20
1/80
1/320
1/1280
Dilu. of Developing Antiserum

indicating very little inhibition of 19S plaques from such dilutions on. In other words, in using this DA to reveal 7S plaques the desirable dilutions should not be lower than 1/40. On the other hand, the similar study carried out at day 10 after immunization showed that the dilution for optimal development of 7S plaques was about 1/640 (peak of graph). That is to say, a 1/640 dilution of this DA would maximally develop 7S plaques, yet minimally interfering with the appearance of 19S plaques, at least in this mouse anti-SRBC system.

The next step was to test the DA in the allogeneic system. A group of C57BL/6 mice was immunized with SaI cells as described in Materials and Methods. Three weeks later all the animals received a boosting injection of 4×10^7 A spleen cells. At least four animals were killed at intervals after the secondary immunization as indicated in FIGURE 10, and the spleen cells were then pooled and assayed. It can be seen that a peak response occurs at day 6 after the secondary antigenic stimulation and that this response falls abruptly by day 8. While the DA is definitely inhibiting at 1/640, higher dilutions do not seem to reveal any more plaques than those detected without DA, except for the experiment at day 6 which indicates a modest increase of plaques with the 1/1280 dilution of DA. This absence of a significant number of indirect plaques could be interpreted to mean that no 7S PFC

FIGURE 10. Kinetics of appearance of plaque forming cells after a secondary immunization of C57BL/6 mice with A tissue cells (see text). Each point represents an average of triplicates from a pool of at least four spleens. Zero (0) concentration means an absence of DA from the culture medium. RC was used.


280
200
120
40
PFC PER 2x10^7 SP. CELLS
d. 6
d. 4
d 3
d. 8
1/40
1/160
1/640
1/2560
0
DILU. OF DEVELOPING ANTISERUM

were present in the spleen of these animals immunized with alloantigens. However, this interpretation is unlikely and is in contrast with the fact that 7S alloantibodies are almost always present in secondary responses to alloantigens (Moller, G., 1966). Winn (1965) and Haughton and McGehee (1969) have shown that rabbit C, in contrast with guinea pig C, works quite efficiently with low molecular weight (7S) antibodies. In conclusion, it is probable that 7S plaques are detected with RC in the absence of any developing antiserum which would help little, if at all, in the development of more 7S plaques. The following experiment was designed to gather more evidence in support of this possibility.

(c) An Attempt to Differentiate 19S from 7S Plaque Forming Cells by Means of Concanavalin A (Con A): Concanavalin A (con A) is a phytoagglutinin or lectin isolated from Jack Bean meal (Agrawal and Goldstein, 1967) and has been shown to precipitate several classes of serum glycoproteins (Goldstein and Iyer, 1966), among which IgM has been identified by immunoelectrophoresis (Leon, 1967). On the other hand, IgG molecules are little affected, presumably due to the lack or non-availability of non-reducing mannosyl and N-acetylglucosaminyl residues for which con A is specific (Goldstein and Iyer, 1966). Nordin et al. (1969) first attempted to make use of this phenomenon to

differentiate 19S from 7S PFC. The experiments reported here were based on the method described by Nordin et al. (1969). Again the validity of the approach was first tested by investigating the effects of con A on plaque formation in the well studied mouse anti-SRBC system. The design of the experiment which is summarized in FIGURE 11 was the following: (1) C57BL/6J mice were immunized with 0.2 ml of 25% SRBC injected intraperitoneally , and spleens were removed 7 or 8 days later in two different experiments, i.e., at a time when 19S and 7S PFC are expected to be present more or less in the same number according to Sterzl and Riha (1965). The spleen cells were pooled and plated for the determination of PFC. (2) The number of indirect 7S and direct 19S plaques was determined with the aid of DA using the method described in Materials and Methods (page 62). (3) The other plates were treated with con A which was expected to inhibit 19S plaques without interfering with the detection of 7S plaques. These plates were divided into three groups: group C was treated with DA and C to develop indirect plaques, group D was treated with C only to develop any direct plaques which had not been inhibited by con A, and group E was treated with α-CH_3-D-mannoside to reverse the effects of con A and then with C to develop direct plaques. If the assumption of inhibition of 19S plaques by con A with no interference with 7S plaque

Experiments with the Use of Concanavalin A (CON A):

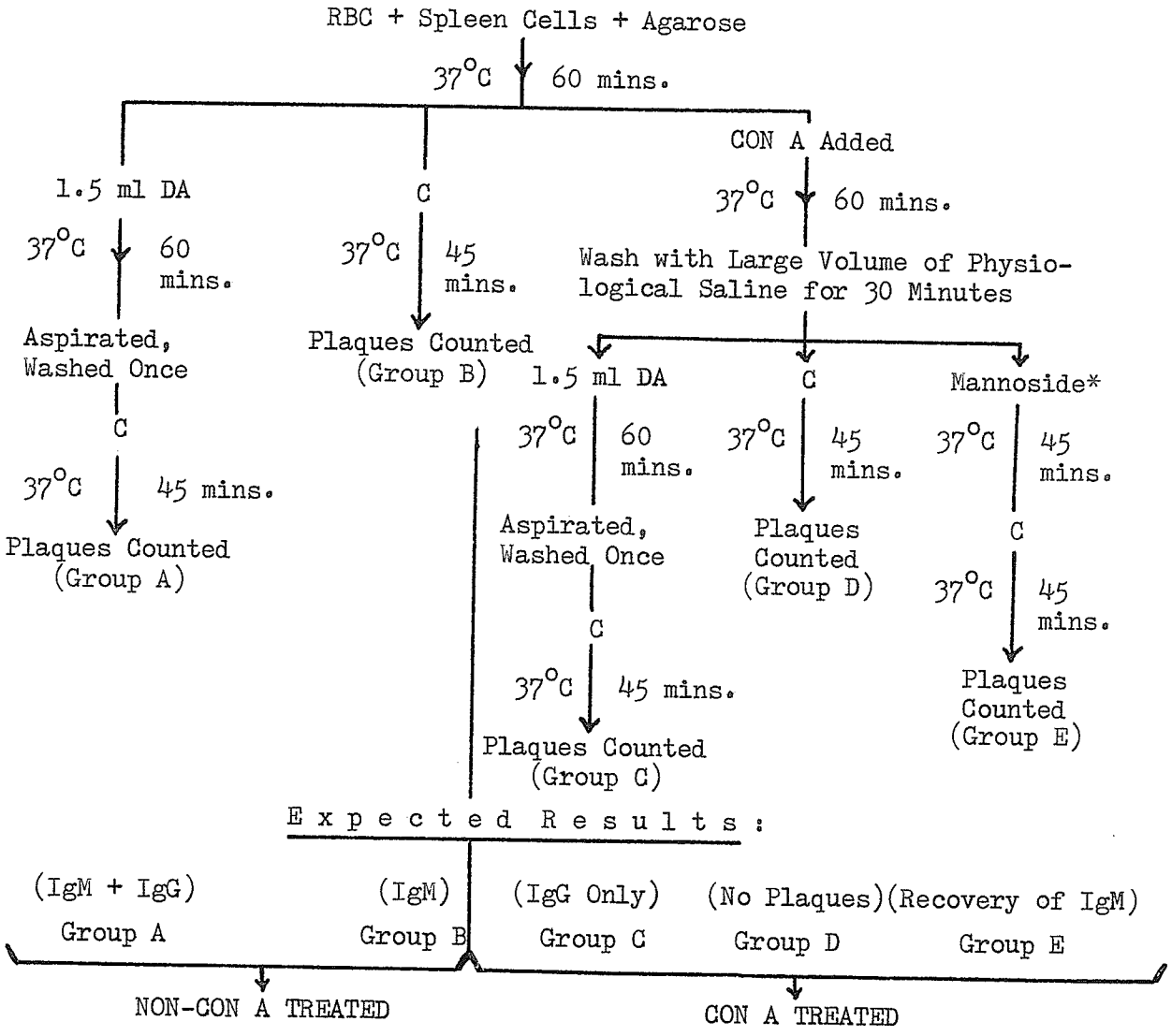


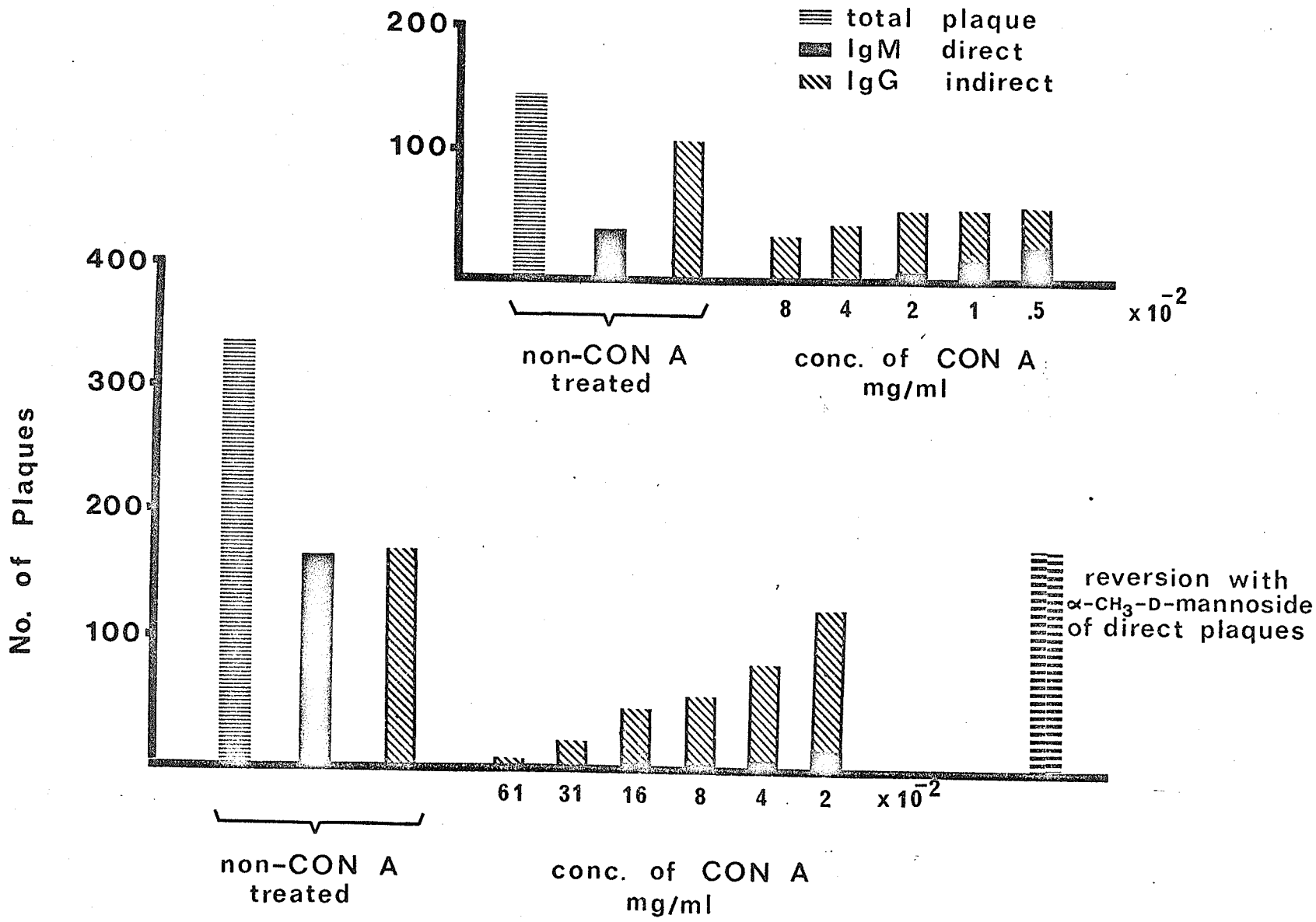
FIGURE 11. This experiment was designed to test the ability of CON A to differentiate 19S from 7S antibody producing cells. α-methyl-D-mannoside was used to recover the 19S plaques annihilated by the previous CON A treatment, thus confirming the specificity of CON A. GPC and RC were used in the mouse anti-SRBC, and mouse allogeneic systems respectively. (Method modified from Nordin et al., 1971)

* Used only in the mouse anti-SRBC system

formation is correct, one would expect to detect: (1) in group C the same number of 7S indirect plaques as calculated by subtracting the number of direct plaques from the total number of plaques detected with the use of DA in the absence of con A, (2) in group D, no plaques, (3) in group E the same number of direct plaques as in group B. Obviously, to obtain these results, not only the afore-mentioned basic assumption should be correct, but also the optimal concentration of con A should be determined. The results of this experiment are reported in FIGURE 12, where the number of 7S plaques was calculated by subtracting the number of 19S plaques (Group B) from the developed plaques (Group A). It can be seen that low con A concentrations (up to 2×10^{-2} mg/ml) did not effectively inhibit all the 19S plaques but yet 7S plaques did not reach the expected level either (i.e., the 7S level calculated in the non-con A-treated group). There were concentrations at which no 19S plaques appeared, but the 7S level was so low that it is obvious that con A somehow interfered with 7S plaque revelation as well. The specificity of the effect of con A is demonstrated by the complete reversion of direct (19S) plaques by using the sugar α-methyl-D-mannoside to recover the 19S plaques.

Experiments with con A of the same design were repeated in the allogeneic system. A group of C57BL/6 mice was immunized with A

FIGURE 12. Potential of concanavalin A in the selective destruction of 19S plaques in the mouse-SRBC system. Plaques are expressed per plate. The number of expected 7S plaques is calculated by subtracting the number of direct plaques from the number appearing on the indirect plates (19S + 7S). Results are arithmetic means of triplicates from a pool of at least three spleens. The two (upper and lower) histograms represent two separate experiments. GPC was used.


200
100
total plaque
IgM direct
IgG indirect
8 4 2 1 .5
x 10⁻²
non-CON A treated
conc. of CON A mg/ml
400
300
200
100
No. of Plaques
reversion with α-CH3-D-mannoside of direct plaques
61 31 16 8 4 2
x 10⁻²
non-CON A treated
conc. of CON A mg/ml

SaI tumor cells and boosted with a dose of 4×10^7 A spleen cells three weeks later, as described elsewhere. PFC were then assayed on day 5 after this secondary immunization. The results are tabulated in TABLE VI (please refer again to FIGURE 11). The results showed that plaques appeared in Group D, suggesting that RC developed some 7S plaques, even in the absence of DA, or that the con A did not inhibit all 19S plaques. On the other hand, the sequential use of con A and DA (group C) resulted in more plaques than con A only, thus indicating that DA in these conditions may have developed some 7S plaques not revealed by the direct assay.

In conclusion this part of the study indicated that: (1) con A interferes with the detection of some 7S plaques in addition to blocking at the appropriate concentration all 19S plaques. This result is at variance with the results of Nordin et al. (1969); (2) in the present experiments, there was no concentration of con A which allowed the development of all 7S plaques while inhibiting at the same time all 19S plaques. Therefore con A in the conditions used in the present study was not suitable for a precise differentiation of 7S and 19S PFC. One possible reason for the apparent incompatibility of DA and con A is that some of the anti-mouse gamma G antibodies in DA, being 19S molecules themselves, reacted with con A, with the loss of at least some of the developing activity; (3) for these reasons, the

NUMBER OF PLAQUES

GROUPS

Experiments	A (with DA)	B (without DA)	C (with DA)						D (without DA)					
	Non-CON-A treated		Conc. of CON A overlaying the top layer (mg/ml)											
			61	31	16	8	4	2	61	31	16	8	4	2
1	88.0 (79-96)	80.0 (77-85)	0	5.0 (4-6)	7.7 (7-8)	17.7 (15-23)	21.0 (17-22)	15.0 (12-17)	0	0	1.7 (1-2)	4.7 (4-5)	6.3 (5-7)	4.3 (3-5)
2	86.0 (83-90)	65.0 (60-69)	0	8.0 (7-9)	10.0 (10-10)	11.3 (10-12)	20.0 (18-22)	19.6 (17-22)	1 (0-2)	0	3.0 (2-4)	5.0 (4-6)	4.3 (4-5)	5.7 (5-6)
Average	87.0	72.5	0	6.5	8.9	14.5	20.5	17.3	0.5	0	2.4	4.9	5.3	5.0

TABLE VI. Test of concanavalin A in the selective inhibition of 19S plaque formation in the mouse allogeneic system. A, B, C and D refer to groups indicated in FIGURE 11. Results are arithmetic means of triplicates from a pool of five spleens in two separate experiments. Average of these are shown at the bottom line. Ranges are in parentheses. RC was used.

results of the experiment with con A in the allogeneic system (as well as in the xenogeneic system reported earlier) were not conclusive in establishing a quantitative relationship between 7S and 19S plaques, but nevertheless strongly suggested that RC is capable of revealing some 7S plaques by itself (i.e., in the absence of DA).

(C) Study of the Specificity of the Plaque Method

The antibody plaque technique described in the current investigation has been demonstrated to be highly sensitive and presumably to detect both 19S and 7S plaque forming cells. As for any other immunological methods, its specificity should also be demonstrated. Specificity of plaque formation may be demonstrated in several ways. Firstly, when normal or immune spleen cells are plated with syngeneic target RBC, one would expect absence of plaque formation, since production of antibody to syngeneic tissue cells would be observed only in the case of autoimmune conditions. Secondly, normal (non-immune) spleen cells may or may not contain PFC against allogeneic target RBC, depending on whether or not environmental antigens cross-reacting with the concerned alloantigens (Rapaport et al., 1968) had been presented to the animals. If this is the case, some 'background' plaques would be expected from 'normal' animals. As is obvious, such plaque formation does not necessarily contradict the hypothesis of specificity.

TABLE VII. H-2 chart. Condensed from Klein and Shreffler (1971). Only those alleles and the corresponding specificities involved in the present study were shown.

H - 2 CHART

(Condensed from Klein & Shreffler, 1971)

H - 2 CHROMOSOME SYMBOL	H - 2 K REGION		H - 2 D REGION	
	PRIVATE	PUBLIC	PUBLIC	PRIVATE
	11 17 23 25 31 33	1 3 5 8 34 35 36	1 3 5 6 13 27 28 29 35 36 41 42 43	2 4 30 32
a	11 -- 23 25 -- --	1 3 5 8 -- -- --	- 3 - 6 13 27 28 29 35 36 41 42 43	- 4 -- --
b	-- -- -- -- -- 33	- - - - - -- 35 36	- - 5 6 -- 27 28 29 -- -- -- -- --	2 - -- --
d	-- -- -- -- 31 --	- - - 8 34 -- --	- 3 - 6 13 27 28 29 35 36 41 42 43	- 4 -- --
k	11 -- 23 25 -- --	1 3 5 8 -- -- --	*1 *3 *5 - -- -- -- -- -- -- -- -- --	- - -- 32
q	11 17 -- -- -- --	- - 5 - 34 -- --	*1 *3 - 6 13 27 28 29 C C C C C	- - 30 --

* = modified Ag

C = weak cross-reactivity

Thirdly, immune spleen cells may form variable numbers of plaques with allogeneic RBC from a strain unrelated to the donor of the immunizing tissues according to the number of alloantigens shared between the two unrelated strains, or to the presence of different but cross-reacting alloantigens. Cross-reactivity between two strains may be due to either non-H-2 or H-2 alloantigens. In the H-2 case, some predictions may be made on the basis of the H-2 chart (TABLE VII), at least for congenic strains, which are genetically identical except at the H-2 region (Snell, 1948; Snell and Bunker, 1965; Snell and Stimpfling, 1966). As is obvious, even if the number of specificities common between the strains may be known, their immunogenicity (or "strength") is usually unpredictable and, therefore, such estimations cannot be quantitative. Finally, the specificity of the hemolytic plaque technique may be tested by inhibiting plaque formation with soluble alloantigens incorporated in the top layer. The soluble antigens will combine with the alloantibodies as released by the PFC, thus preventing them from sensitizing the target RBC. Complete inhibition (no plaques) would be expected if the specific soluble antigens (i.e., antigens carried on the cells which had been used to immunize the spleen cell donor and present on the target

RBC) are used. Partial inhibition (fewer plaques) would be expected with alloantigens different from, but cross-reacting with the specific ones; and no inhibition would be expected with alloantigens syngeneic or non-cross-reacting with the spleen cell donor.

a) The Use of Normal and Immune Cells with Syngeneic and Allogeneic RBC as Targets: In the following experiments aimed at verifying the specificity of the plaque method, immune spleen cells were obtained from C57BL/6 mice immunized with A tissue cells 4-5 days after the secondary immunization as described in Materials and Methods. TABLE VIII reports a group of data demonstrating some aspects of the specificity of the present plaque method. It is clear that the results are in accordance with the expectation that normal or immune spleen cells do not contain PFC against syngeneic target RBC in a strain like C57BL/6 which does not present a significant incidence of auto-immune phenomena. Also, normal (non-immune) spleen cells did not contain any PFC against allogeneic target cells: no plaques were observed with normal C57BL/6 spleen cells and A target RBC. In other words, in this allogeneic system there are no background plaques which are commonly observed in other systems (Jerne and Nordin, 1963; Fuji et al., 1971). This may indicate that

DONOR --------> RECIPIENT

A --------> C57BL/6

C57BL/6 spleen cells	Experiment	Target RBC	
		A	C57BL/6
NORMAL	1	0	0
	2	0	N. D.
	3*	0	N. D.
IMMUNE	1	42.0 (38-49)	0
	2	52.0 (50-55)	0
	3*	60.3 (55-67)	0

TABLE VIII. Specificity of the plaque method. Normal (C57BL/6) and immune (C57BL/6 anti-A) spleen cells were plated with allogeneic (A) and syngeneic(C57BL/6) RBC as targets. Results are arithmetic means of triplicates. Ranges are in parentheses. Spleen cells were from a pool of at least three spleens. RC was used. Results are PFC per 2 x 10^7 spleen cells.

N.D. : Not Done

* Performed independently

either the unimmunized animals had never before encountered any environmental antigens cross-reacting with the concerned alloantigens, or that the immune response to such antigens was so weak that it escaped detection with the plaque method.

b) The Use of 'Third Party' RBC as Targets: The specificity tests were further complemented with the use of allogeneic RBC as targets from strains unrelated to the donor ('third party') of the immunizing tissues. Immune spleen cells were from C57BL/6 animals 4 or 5 days after a secondary immunization with A spleen cells as described in Materials and Methods. As donors of indicator RBC, animals of the following strains were used: A, C57BL/6, AKR, CBA, DBA/1, DBA/2 and B6AF1.

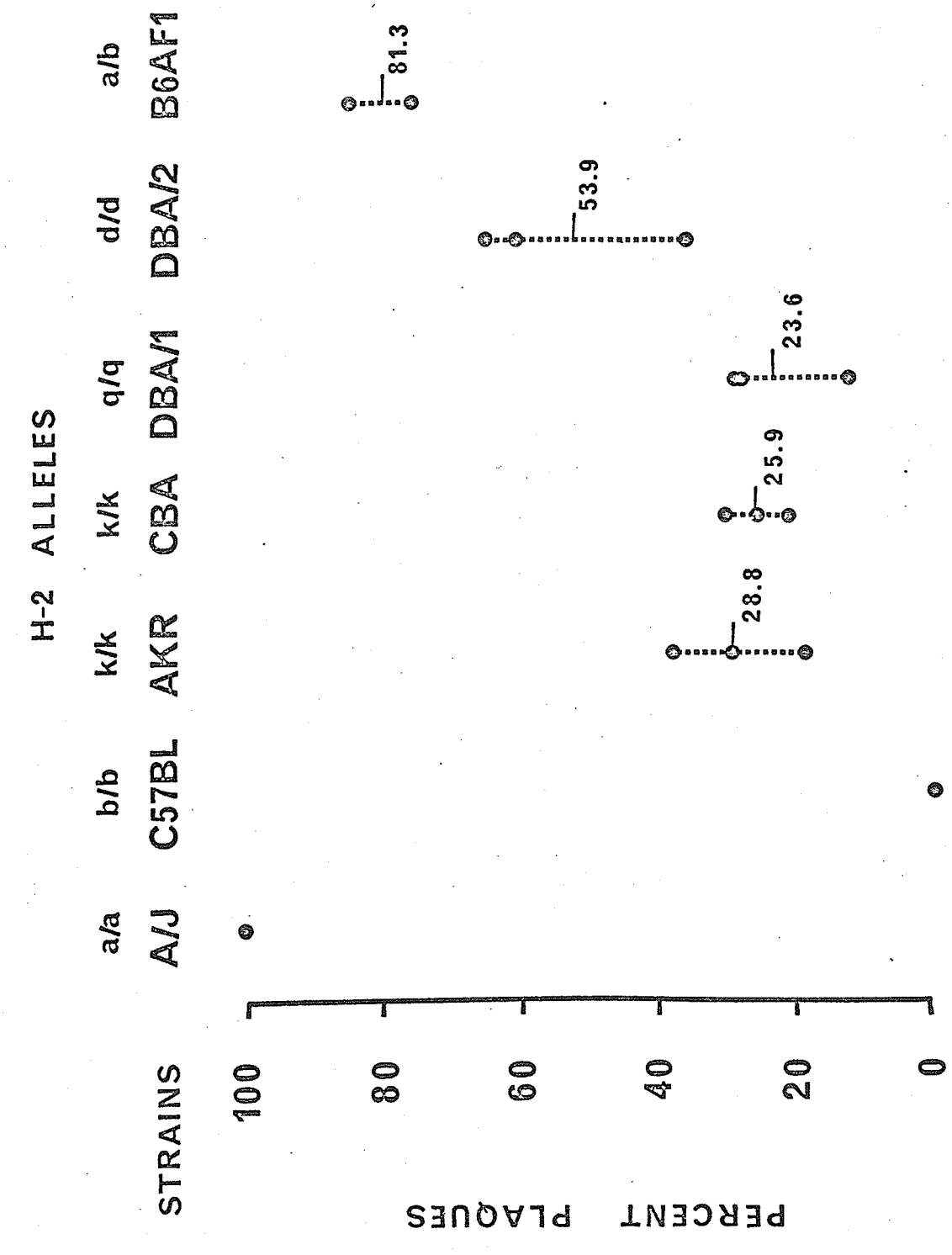
The results of such a study are presented in FIGURE 13. The data were expressed as percent plaques calculated according to the formula:

$$P = \frac{N_T}{N_A} \times 100$$

where P = percent plaques; N_A = number of plaques appearing on plates containing target RBC of the immunogen type, i.e., strain A ($H\text{-}2^a$); and N_T = number of plaques appearing on plates containing the test target RBC from different mice strains. Consequently, with A RBC as targets, percent plaques is always 100. It can be seen that while the percent plaques is always zero with the recipient as targets, showing no immune response against syngeneic target RBC, the values vary with the different allogeneic target

FIGURE 13. Specificity study of the hemolytic plaque method. Number of plaques with target RBC from different strains was expressed as the percentage of the number of plaques obtained with the specific A RBC. The strain donors of RBC are indicated with their respective H-2 genotypes. Each point represents the arithmetic mean of triplicates of a pool of at least four spleens. The bars indicate the arithmetic means of three separate experiments (only two for the B6AF1* case). RC was used.

* B6AF1 = (C57BL/6 X A)F1 hybrids


PERCENT PLAQUES
100
80
60
40
20
0
STRAINS
H-2 ALLELES
a/a
A/J
b/b
C57BL
k/k
AKR
k/k
CBA
q/q
DBA/1
d/d
DBA/2
a/b
B6AF1
81.3
53.9
23.6
25.9
28.8

RBC from strains known to carry H-2 alleles possessing some specificities in common with the H-2^a antigens present in the immunizing tissues of strain A. Thus, with both AKR and CBA strain RBC (both carrying the H-2^k allele), which carry 7 of the 15 immunizing antigens (please consult the H-2 chart, TABLE VII), percent plaques were 28.8 and 25.9 respectively. In other words, the number of plaques obtained with these target RBC was about 27% of those appearing on plates with the specific A RBC. Similarly, DBA/2 (H-2^d) target RBC which carry 9 out of the 15 immunizing antigens gave 53.9 percent plaques. Likewise, DBA/1 (H-2^q) RBC gave 23.6%. It is interesting to note that B6AF1 RBC, which are known to carry a full complement of the immunizing A antigens (B6AF1 = (A x C57BL/6)F1), yeilded only 81.3% plaques.

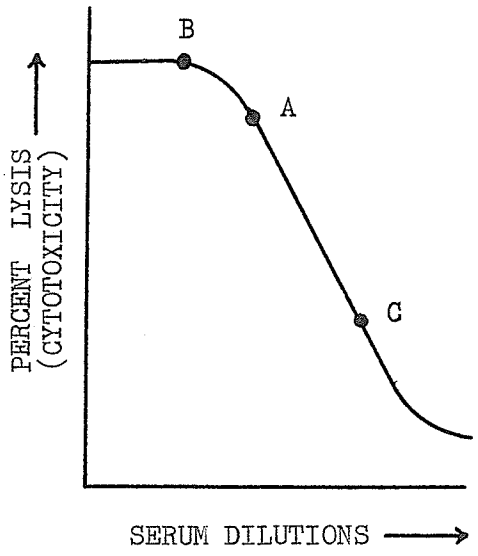
It should be noted that H-2 as well as non-H-2 differences are involved in the present combination, since A and C57BL/6 are not congenic strains; nor any of the other strains used to donate RBC were congenic with C57BL/6. Therefore, the observed cross-reactivity cannot be attributed to H-2 antigens alone, and non-H-2 antigens may play a role as well. For this reason, the interpretation of these results will be discussed in a later section when congenic strains

are considered.

(c) Inhibition of plaque Formation with Soluble Antigens:

(1) Cytotoxic Tests and Cytotoxic Inhibition Tests: In order to investigate further the specificity of the plaque method, it was tested if soluble alloantigens incorporated in the top layer would inhibit plaque formation, and to what extent. For this reason, soluble alloantigens were prepared using the 3M KCl hypertonic treatment of lymphoid cells (Reisfeld et al., 1970; Reisfeld and Kahan, 1971). However, since these antigenic preparations had to be titrated by means of the cytotoxic inhibition tests, some alloantisera were first prepared and their titres measured to determine the proper dilutions to be used in the cytotoxic inhibition test. The rationale of the experimental design is as follows. The antigenic content of an antigenic preparation is measured by its ability to inhibit the cytotoxic effect of a specific alloantiserum of known activity. To do this, the dilution of the antiserum in question has to be carefully chosen. The best dilution to be used should be one corresponding to a cytotoxicity close to (but lower than) 100% lysis of the specific target cells. In other words, a dilution corresponding somewhat to the neighbourhood of

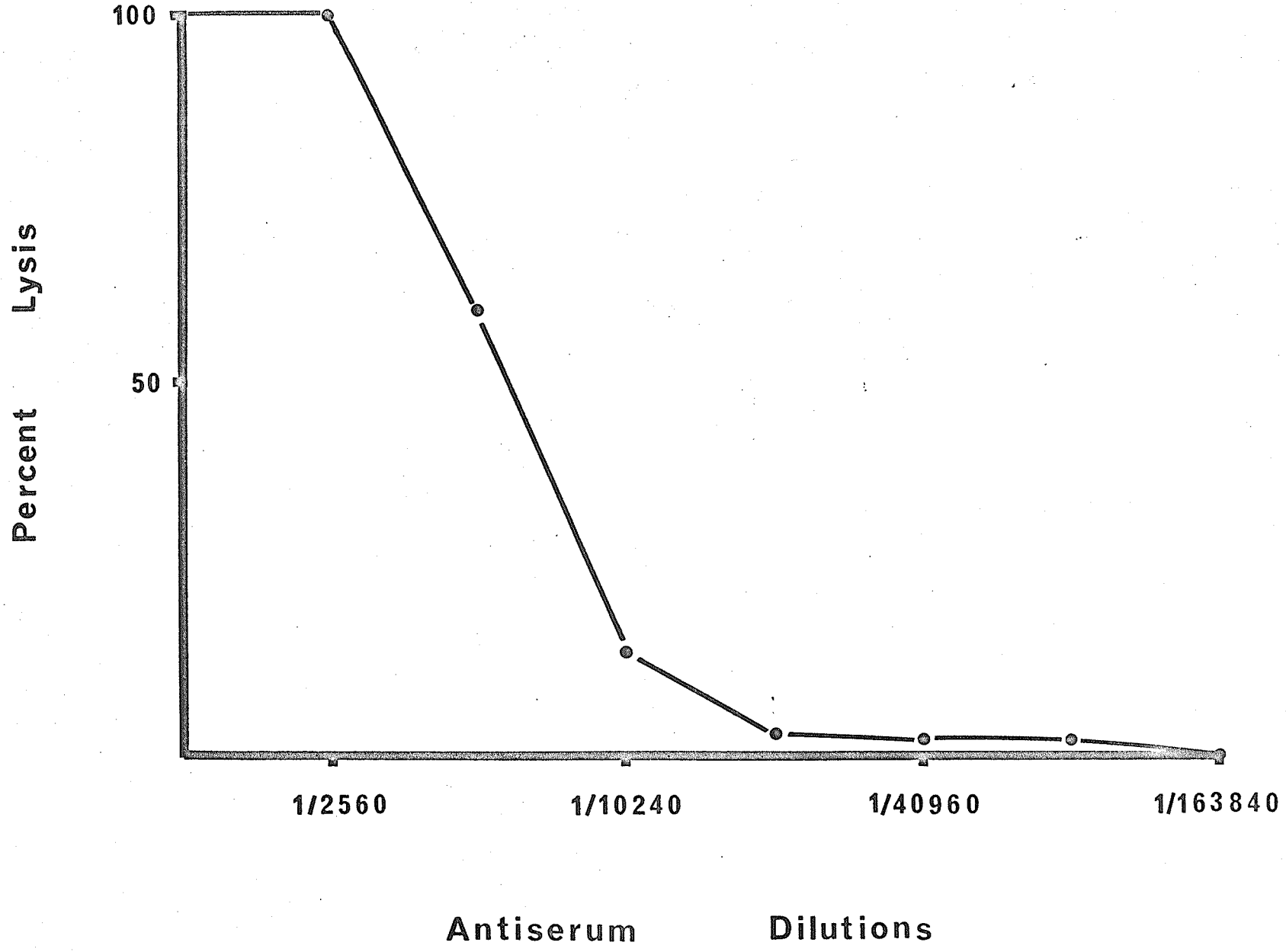
point A on the hypothetical cytotoxic curve in the accompanying figure should be chosen. A higher concentration of serum too close to, or in, the plateau (B) would give a low sensitivity since a relatively large amount of antigenic material would be required to inhibit the cytotoxicity of the antiserum. A point too low down the curve (C), on the other hand, would allow a very sensitive detection of the antigen, but not a quantitative measurement of it, since a small amount of antigen could readily inhibit completely the cytotoxicity of the serum.



For establishing the cytotoxic curves cytotoxic tests using ^{51}Cr-labeled target cells were employed to titrate the alloantisera.

(i) Cytotoxic Tests for Titration of Alloantisera: Two antisera, C57BL/6 ($H\text{-}2^b$) anti-A ($H\text{-}2^a$) and A anti-C57BL/6, were titrated using ^{51}Cr-labeled donor lymph node cells as target and guinea pig serum as C source. The C57BL/6 anti-A serum

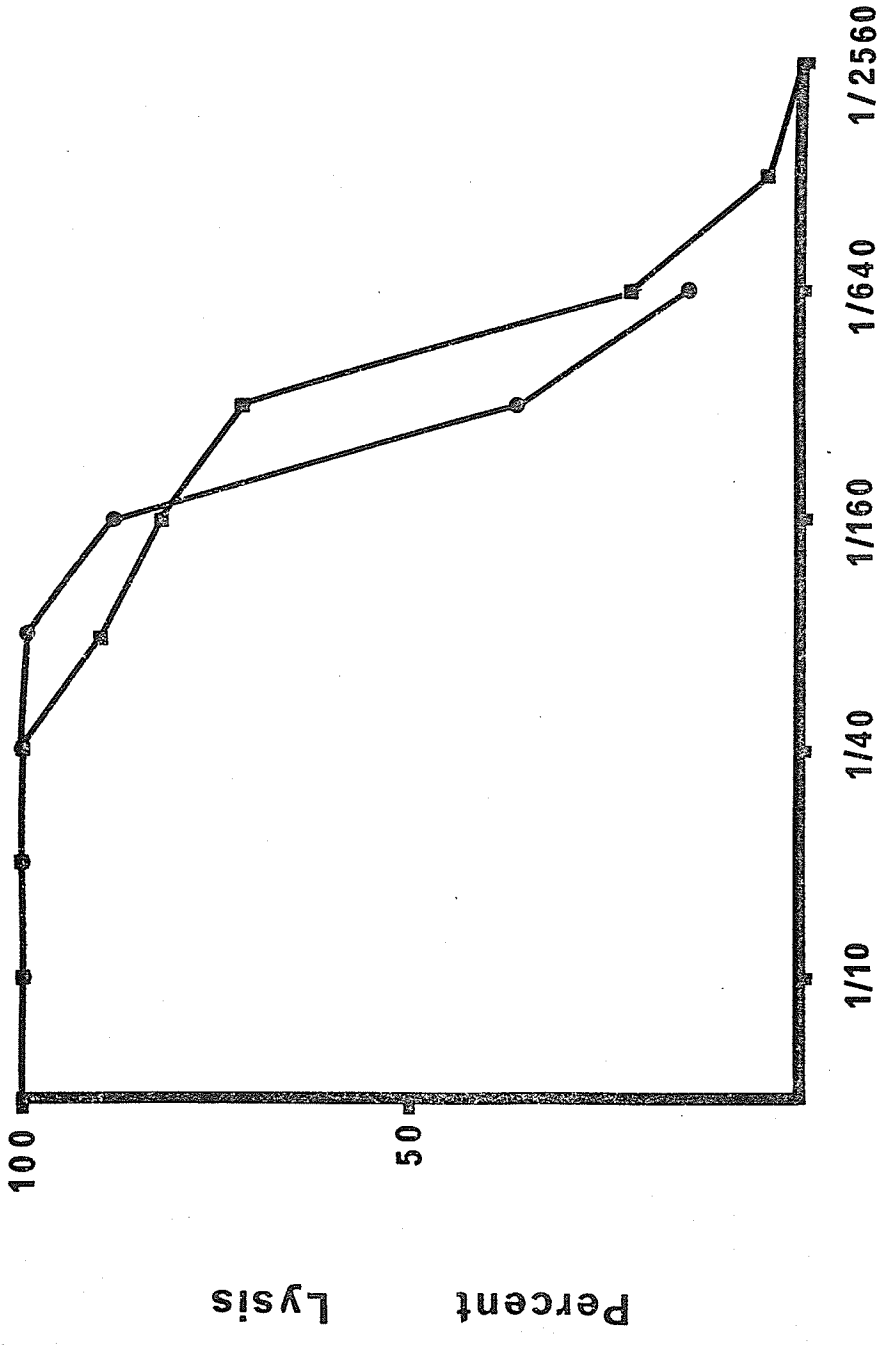
FIGURE 14. Cytotoxicity test for the titration of C57BL/6 anti-A alloantiserum, using ^{51}Cr-labeled A lymph node cells as targets. Each point represents the arithmetic mean of triplicates performed in a single experiment. GPC was used.


100
50
Percent Lysis
1/2560
1/10240
1/40960
1/163840
Antiserum Dilutions

was prepared by multiple weekly injections of SaI (a sarcoma indigenous to strain A) cells into young adult C57BL/6 animals intraperitoneally as described in Materials and Methods. For this antiserum, titration was done immediately before the cytotoxic inhibition test proper (in order to minimize titre variation due to storage of serum). The serum was serially diluted in the wells of a MicroTest II plate. Into these wells were added the specific target cells (strain A lymph node cells) labelled with ^{51}Cr, and the plate was incubated for 20 min. at room temperature. Guinea pig C was then added and the plate was further incubated at 37°C for 30 min., after which the supernatant was taken out and its radioactivity was counted in a well-type gamma scintillation counter. Results of such a titration are reported in FIGURE 14. It can be seen that the plateau begins to subside at the 1/2560 dilution, and minimal cytotoxicity was observed at a dilution of 1/20480. Consequently a dilution of 1/4000 corresponding to about 80% lysis was chosen to be used in the cytotoxic inhibition assay.

The A anti-C57BL/6 antiserum was prepared by injecting A strain mice with 1×10^7 C57BL/6 lymphoid cells (from thymus, spleen and lymph nodes) intraperitoneally the first week, and

FIGURE 15. Cytotoxicity test for titration of A anti-C57BL/6 allo-antiserum, using ^{51}Cr-labeled C57BL/6 lymph node cells as target. Each point represents the arithmetic mean of triplicates. Two separate experiments were performed. GPC was used.

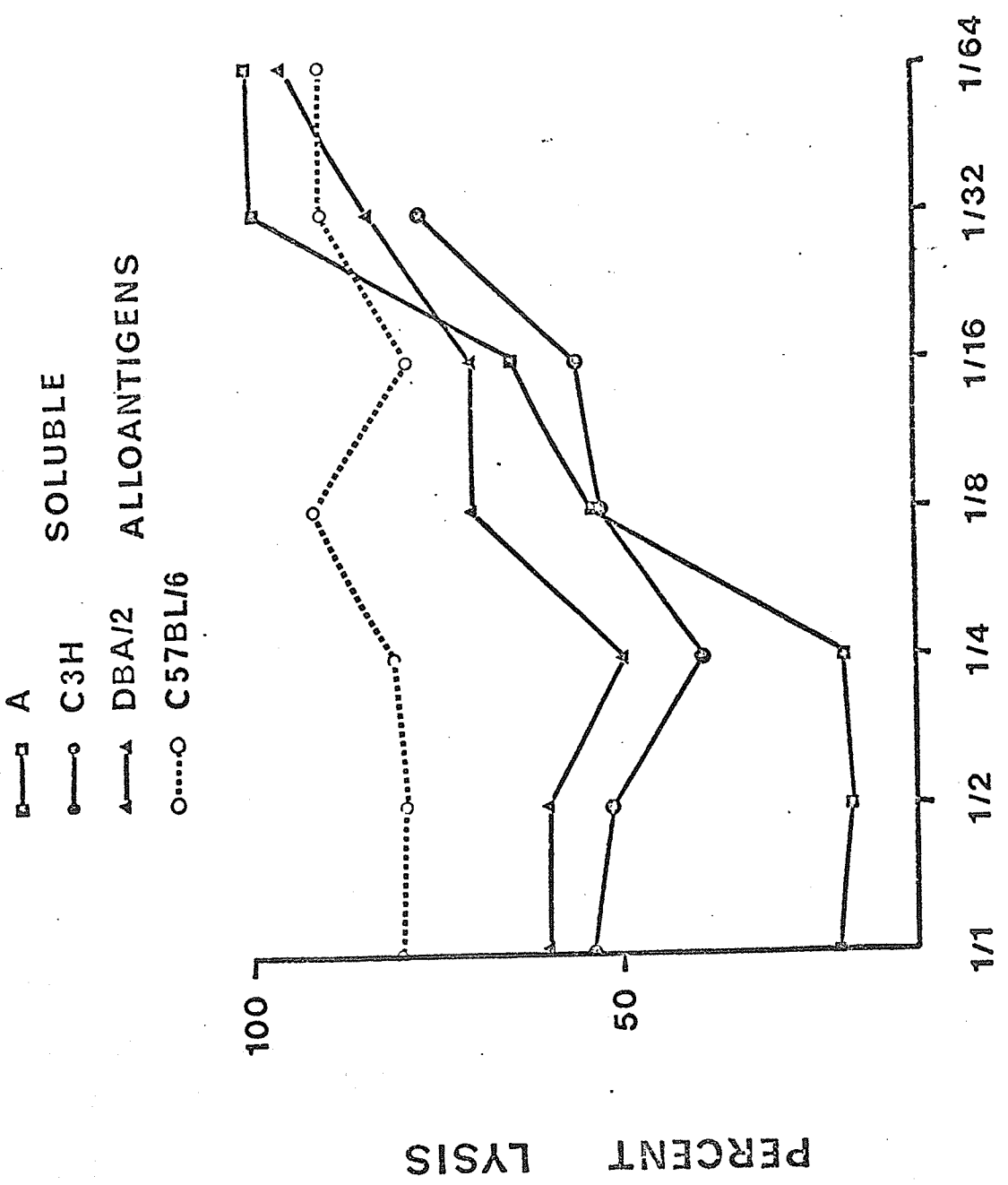

100
50
Percent Lysis
1/10
1/40
1/160
1/640
1/2560
Antiserum Dilutions

2.5×10^7 cells weekly thereafter for seven weeks. The animals were given a month's rest, and then weekly injections of 5×10^7 cells were given intraperitoneally for six more weeks. The results of the titration of this antiserum is shown in FIGURE 15. In this case two titrations were done in two seperate experiments using different batches of target cells (^{51}Cr-labeled C57BL/6 lymph node cells) about one week before the inhibition test. A dilution of 1/160 of this antiserum, which correspond to about 80% lysis, was chosen for subsequent cytotoxic inhibition experiments.

(ii) Cytotoxic Inhibition Tests for Alloantigens:

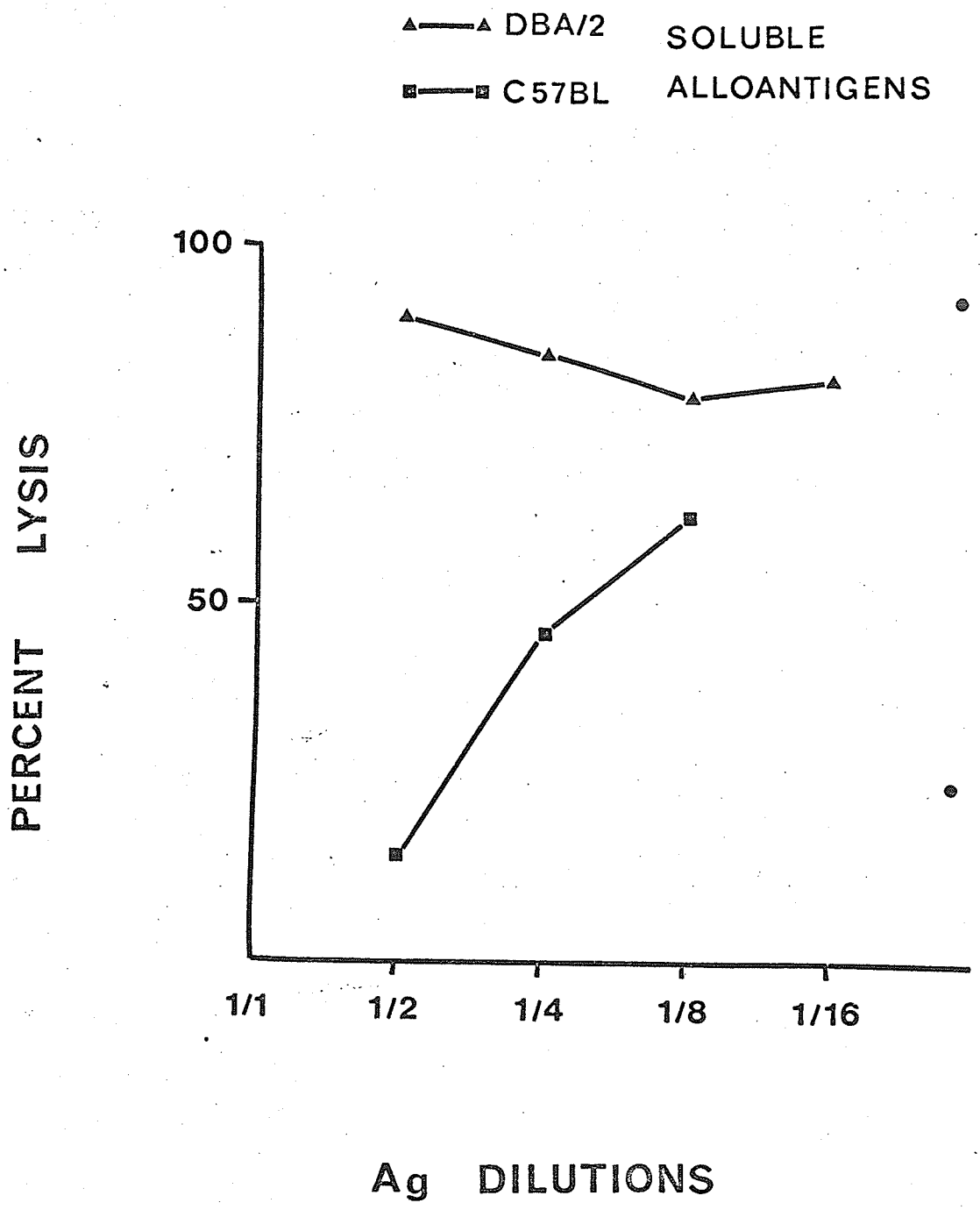
Soluble alloantigens from four strains: A, C3H, DBA/2 and C57BL/6, were prepared using the 3M KCl hypertonic salt extraction method of Reisfeld et al. (1970) and Reisfeld and Kahan (1971), and their antigenicity were measured by their ability to inhibit the cytotoxicity of alloantisera. In one experiment the cytotoxic antiserum used was C57BL/6 anti-A at a dilution of 1/4000 (as explained above), and the target cells were the specific A lymph node cells. Thus, strain A alloantigens, being the specific antigens, should be expected to cause somplete inhibition, while the C57BL/6 antigens, i.e., obtained from the strain producer of the antiserum, would give

FIGURE 16. Inhibition of the cytotoxicity of a C57BL/6 anti-A serum on the specific A target cells by antigenic extracts from four different strains. 100% target cell lysis is represented by the upper bar. The lower bar represents the cytotoxicity induced by the A antigenic preparation (1/1) in the presence of GPC (thus indicating the slightly toxic nature of the preparations in general). Each point represents the arithmetic mean of triplicates performed in a single experiment. GPC was used.


A
C3H
DBA/2
C57BL/6
SOLUBLE
ALLOANTIGENS
PERCENT LYSIS
100
50
1/1
1/2
1/4
1/8
1/16
1/32
1/64
Ag DILUTIONS

no inhibition. On the other hand, by virtue of the common specificities shared between the k (C3H) and d (DBA/2) alleles with the donor a (A) allele respectively, C3H and DBA/2 alloantigens should be able to give partial inhibition. FIGURE 16 shows the results of such a study. The upper bar represents the cytotoxicity level of the antiserum at a 1/20 dilution, and was taken to be 100% lysis (see titration curve, FIGURE 14). It can be seen that up to a dilution of 1/4, the specific A alloantigens gave a complete inhibition while the C57BL/6 antigens, being syngeneic to the strain producer of the antiserum, did not give any significant inhibition, with the cytotoxicity staying at more or less the 80% lysis level. On the other hand, with the cross-reacting DBA/2 and C3H alloantigens, partial inhibition was obtained. The inhibitory property of the antigenic preparations disappeared almost completely at a dilution of 1/32. With these results, therefore, it can be concluded that the alloantigen preparations obtained with the hypertonic salt extraction method induced specific inhibition of cytotoxicity. The other antiserum (A anti-C57BL/6) was expected to contain antibodies against two specificities only, viz., 33 and 2. Since the H-2^d allele does not contain these specificities, alloantigens extracted from the

FIGURE 17. Inhibition of the cytotoxicity of an A anti-C57BL/6 serum on the specific C57BL/6 target cells by antigenic extracts from DBA/2 and C57BL/6 strains. The upper closed circle represents the cytotoxicity of the antiserum in the presence of the syngeneic A antigens and GPC; and the lower one, the cytotoxicity of the DBA/2 antigen extract itself in the presence of GPC. Each point represents the arithmetic mean of triplicates in a single experiment. GPC was used.


DBA/2
C57BL
SOLUBLE
ALLOANTIGENS
100
50
PERCENT LYSIS
1/1
1/2
1/4
1/8
1/16
Ag DILUTIONS

lymphoid tissues of DBA/2 mice ($H\text{-}2^d$) were used as control. The results of this experiment are shown in FIGURE 17. As expected, the DBA/2 alloantigens did not give any significant inhibition, with the cytotoxic curve staying at more or less the 80% level. The C57BL/6 alloantigens, on the other hand, showed highly significant inhibitory effect. The syngeneic A antigens (at a 1/1 dilution) did not affect the cytotoxicity of the A anti-C57BL/6 antiserum (upper closed circle). From these results, it can be concluded that the antigenic preparations had fulfilled all the requirements of specificity.

(2) Inhibition of Plaque Formation with Soluble allo-Antigens: The specificity of the plaque technique was tested by incorporating the soluble antigens described above into the agarose top layer in order to inhibit plaque formation. Alloantibodies that are released from the PFC would react with the alloantigens, and would thus be prevented from sensitizing the target RBC.

A group of C57BL/6 mice were immunized with SaI cells, and three weeks later, boosted with A spleen cells as described in Materials and Methods. PFC were then assayed 4 or 5 days after the secondary immunization with the usual technique, with the exception

TABLE IX. Inhibition of plaque formation by the incorporation of soluble alloantigens into the top layer. Results are arithmetic means of triplicates of PFC per 2 x 10^7 spleen cells from a pool of five spleens. Ranges are in parentheses. RC was used.

AM'T. Ag PER PLATE (mg protein)	ANTIGEN EXTRACT FROM A/J ($H\text{-}2^a$)	C57BL/6 ($H\text{-}2^b$)	DBA/2 ($H\text{-}2^d$)	C3H ($H\text{-}2^k$)
1.10	0	100 (90-109)	0	48 (40-59)
0.55	53 (50-56)	108 (89-128)	58 (51-63)	95 (90-100)
0.28	109 (88-129)	114 (101-121)	63 (46-80)	90 (66-103)
No Ag	129 (98-170)			

that 0.1 ml of antigen (0.28 to 1.1 mg protein) was incorporated into the top layer, and the volumes of the other components in the top layer was adjusted so that the final volume was the same as in the usual plaque assay. Thus, the composition of the top layer was as follows: 0.3 ml 2-fold concentrated HEPES-Hanks', 0.3 ml of 0.83% agarose in double distilled water, 0.05 ml of 25% RBC, 0.05 ml of spleen cells of concentration 2 x 10^8/ml, and 0.1 ml of antigen. TABLE IX shows the results of this inhibition of plaque formation assay. It is clear that syngeneic C57BL/6 antigens inhibited slightly plaque formation, as compared with the control plates containing no antigen in the top layer, probably because of some cytotoxicity of the antigenic preparation itself which was seen also in the afore-mentioned cytotoxic inhibition experiments (pages 120, 122). The specific A alloantigens, on the other hand, completely inhibited plaque formation with 1.1 mg protein per plate, and reverted to the control level if only 0.28 mg was present. Partial inhibition was seen with DBA/2 and C3H alloantigens. It is interesting to note that at 1.1 mg protein DBA/2 antigens were capable of giving complete inhibition, while C3H antigens could only partially inhibit.

(3) The Use of Target RBC from Congenic Strains:

In order to study in more detail the specificity of the plaque technique with respect to H-2 antigens, congenic strains were used as target RBC donors in experiments similar to those described in the preceding section. C57BL/10 (also known as B10) mice were immunized with SaI cells and boosted with the injection of A spleen cells as described for C57BL/6 mice. Target cells with the same H-2 alleles as in the previous experiments were obtained from the strains: B10.A ($H\text{-}2^a$), B10.BR ($H\text{-}2^k$) and B10.D2 ($H\text{-}2^d$), which are congenic with B10. In this way, only H-2 plaques can be detected. On the other hand, if strain A RBC are used as target in this donor-recipient combination, both non-H-2 and H-2 plaques can be detected. The results of this experiment are reported in TABLE X. The number of plaques appearing on plates with target RBC from strain A was again taken as 100%, and the percent plaques for the other target cells was calculated according to the formula: $\frac{N_T}{N_A} \times 100$ (see page 111). It can be seen that with B10.A target RBC, the number of plaques was reduced to approximately 50% of those obtained with the RBC of the immunogen type A. This result clearly indicates that non-H-2 antigens in this A --> B10 combination

PERCENT PLAQUES

Experiment	A H-2^a	B10.A H-2^a	B10 H-2^b	B10.BR H-2^k	B10.D2 H-2^d
1	100 (121.7)	49.3 (60.0)	1.4 (1.7)	1.4 (1.7)	33.7 (41.0)
2	100 (92.3)	56.7 (52.3)	0 (0)	1.4 (1.7)	41.5 (38.3)
3	100 (44.7)	54.8 (24.5)	0 (0)	1.6 (0.7)	51.5 (23.0)

TABLE X. Specificity of the plaque method employing RBC from congenic strains as targets. Number of plaques obtained with target RBC from different strains was expressed as percentage of the number obtained with the specific strain A RBC. Numbers in parentheses are arithmetic means of triplicates (of the numbers of PFC per plate) from a pool of four spleens. RC was used.

contributed to a sizable portion of the total immunogenicity of the A alloantigens recognized by B10. This confirms the similar finding by Hildemann and Pinkerton (1966). The specificity of the assay is well demonstrated by the absence (or the very small number: 1.4, in one of the experiments) of plaques with the syngenic B10 RBC and the smaller number with the B10.D2 ($H\text{-}2^d$) targets when compared with that obtained with the B10.A ($H\text{-}2^a$) RBC as targets. The small number of plaques obtained with B10.BR ($H\text{-}2^k$) RBC similar to the findings reported in FIGURE 13 and TABLE IX indicates that the immune response against antigens controlled by the K end of the $H\text{-}2^a$ chromosome was lower than that against those controlled by the D end of the same chromosome.

The experiment of inhibition of plaque formation by soluble alloantigens was performed with the same A --> B10 (donor-recipient) combination, using specific and cross-reacting soluble alloantigens prepared with lymphoid cells from congenic strains, again using the 3M KCl hypertonic salt method. The results reported in TABLE XI indicate that: (i) only about 50% of the plaques obtained with the specific A RBC are due to H-2 antigens, (ii) these H-2 plaques (i.e., those detected with B10.A target RBC)

TABLE XI. Inhibition of plaque formation by the incorporation of soluble alloantigens in the top layer using RBC from congenic and non-congenic strains as targets. Results are arithmetic means of triplicates of PFC per 2×10^7 spleen cells from a pool of five spleens, with the ranges in parentheses. RC was used.

ALLO-ANTIGENS		NIL		B10.A		B10.BR	B10.D2	SYNGENEIC B10
TARGET RBC		A	B10.A	A	B10.A	B10.A	B10.A	B10.A
EXPERIMENT	1	77.7 (67-88)	47.0 (42-50)	21.7 (19-25)	0.3 (0-1)	41.0 (38-43)	8.0 (7-9)	39.3 (38-40)
	2	88.3 (84-92)	45.0 (42-48)	26.7 (25-28)	0.3 (0-1)	34.7 (33-36	5.0 (4-6)	37.0 (35-39
		a	b	c	d	e	f	g

are completely inhibitable with soluble B10.A alloantigens, and (iii) most of the H-2 plaques are due to antigens of the D-end of the $H\text{-}2^a$ chromosome as shown by the almost complete inhibition of the H-2 plaques with B10.D2 alloantigens and the very low inhibitory activity of B10.BR alloantigens.

The slightly fewer number of plaques obtained with the syngeneic B10 antigens than those obtained with no antigens incorporated in the top layer (columns g and b) can be easily explained by the fact that the antigenic preparations as prepared by the 3M KCl hypertonic salt method were slightly cytotoxic, as demonstrated by the data in the cytotoxic inhibition experiments (FIGURES 16 and 17), thus killing some of the PFC in the first place.

The plaque assay had thus been shown to fulfill even the most stringent tests of specificity.

DISCUSSION

As discussed in LITERATURE REVIEW, although literature concerning the cell types involved in the allograft rejection phenomenon is abundant, clearcut data leading to the resolution of this problem are still lacking. Of the two types of immune responses which may be demonstrated in relation to the rejection of allografts, cell-mediated immunity has been much more enthusiastically studied and consequently better understood. Two types of assays for detecting antibody at the cellular level have been developed for studies in transplantation immunity; viz.: the agar plaque technique (e.g. Pinkerton and Hildemann, 1966; Nordin et al., 1971), and the rosette technique (Micklem et al., 1970). While the latter most likely detects hemagglutinating antibody producing cells (see page 28), the agar plaque technique detects plaque forming cells which produce cytolytic antibodies, i.e., hemolytic antibodies (hemolytic plaques) or cytotoxic antibodies (plaques with nucleated cells). The agar plaque technique for detecting antibody producing cells in the mouse allogeneic system has been employed using target cells of different origins: red blood cells (Hildemann and Pinkerton, 1966), lymphoid tissue cells (Fuji et al.,

1971) and neoplastic cells (Fuji et al., 1971; Nordin et al., 1971), among which only the red blood cells possess the desirable properties of being relatively homogeneous and easily accessible, in addition to the fact that they carry most of the H-2 and non-H-2 specificities (Snell and Stimpfling, 1966). Another distinctive feature of RBC is that they can be available from any mouse strain, while lymphoblastic and neoplastic cells are not always available, making strain combinations quite restricted for study. Thus to render the method more widely applicable to any desirable strain combination, the use of RBC as target cells seems desirable.

As has been noted, the method by Hildemann and Pinkerton (1966) has a low sensitivity and was difficult to reproduce. The aim of the study reported in this thesis was to develop a hemolytic plaque technique of higher sensitivity and with a general applicability to studies of transplantation immunity.

(A) The Sensitivity of the Plaque Assay: As reported by Hildemann and Pinkerton (1966), the plaques formed in the allogeneic system are smaller than those obtained in xenogeneic systems, therefore, the investigation began with the consideration of some physical and geometric factors that may affect the visibility of the plaques.

Firstly, the amount of RBC in the top layer was reduced so that antibody molecules released from the PFC could form plaques as large as possible without any loss of resolution. Next the volume of the top layer was also reduced so that plaques would not be masked by the thickness of the RBC layer.

The technique which was finally adopted resulted in a thickness of 0.42 mm of the top layer, as compared with 0.88 mm and 0.89 mm in the methods described by Jerne and Nordin (1963) and Hildemann and Pinkerton (1966), respectively.

It has been reported that with RC, mouse alloantibody has a higher cytolytic efficiency than with GPC (Winn, 1965; Haughton and McGehee, 1969). The results reported in FIGURE 1 showed that this is also true in the present hemolytic plaque tehnique, as shown by the higher plaque counts obtained with RC in the allogeneic system, while GPC was definitely more efficient in the mouse anti-SRBC system. These findings are in complete agreement with the observations by Winn (1965) and Haughton and McGehee (1969). However, no attempt was made to elucidate the mechanism by which RC effects the higher cytolytic activity. Nonetheless, the possibility that this is due to the presence of natural anti-mouse antibody in the rabbit serum has been excluded (see below).

The inclusion of a xenogeneic rabbit anti-mouse RBC antiserum

(RAMRBC) in the assay system was originally intended to induce a synergistic effect on the development of plaques which would have otherwise escaped attention whenever the other allogeneic hemolytic antibodies were present on the surface of target RBC at a concentration too low for lysis. In actual fact, RAMRBC, instead of increasing, decreased the number of plaques (TABLE II). Since the addition of RAMRBC mimics the presence of natural anti-mouse antibodies, it is only logical to use a rabbit serum freed, as far as possible, of natural antibodies which are almost always present in rabbit sera (Boyse et al., 1970). This precaution was justified by the results reported in TABLE III that absorbed rabbit sera in two independent experiments were shown to enhance, albeit to a small extent, plaque formation as compared with unabsorbed sera.

With the technique thus developed, it was possible to detect a peak response of 115 PFC per 1 X 10^7 spleen cells at day 6 after a secondary immunization (FIGURE 10), as compared with 26.6 PFC in a comparable study by Hildemann and Pinkerton (1966) in primary responses.* When compared directly with the method developed by Nordin et al. (1971).who employed tumor cells instead of RBC as targets, the present mehod was shown to be able to detect ten times as many as PFC (TABLE V).

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* Although the comparison between a primary response (as studied by Hildemann and Pinkerton) and a secondary response (as studied in this thesis) is obviously not valid experiments performed after the completion of this thesis showed that the present technique detects about twice as

(B) Plaques of the 19S and 7S Types: It has been established that with the use of GPC it is possible to detect only PFC producing 19S antibodies, while for the development of IgG plaques it is necessary to add an anti-gamma G antiserum (Dresser and Wortis, 1965; Sterzl and Riha, 1965). A developing antiserum such as the one adopted in the present study, however, helped little, if at all, in developing additional plaques in the mouse allogeneic system. However, this does not mean that no 7S PFC was present in the spleens of these animals since it has been shown that 7S alloantibodies are almost always present in secondary responses. It is reasonable to assume that 7S plaques were developed by RC alone, since RC, in contrast with GPC, has been shown to work quite efficiently with low molecular weight antibodies (Winn, 1965; Haughton and McGehee, 1969). The experiments with Concanavalin A (con A) favored this conclusion. Thus, plates that had been treated with con A but never with DA, did show some plaque formation. However, a comparison of these results with those obtained with the use of DA suggested that some 7S plaques were not developed with RC alone but required the addition of DA.

It is unfortunate that the con A employed in the present study did not selectively inhibit 19S plaque formation. The reasons

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many plaques as the technique of Hildemann and Pinkerton.

for the difficulty of developing plaques with DA in the presence of con A may be several. For example, despite extensive washings of the agarose plates after treatment, residual con A in the Petri dishes may have reacted with anti-mouse gamma G antibodies of the 19S class present in the DA, with a partial loss of its developing property. As a second possibility, it may be suggested that some complement components may possess carbohydrate moieties capable of reacting with con A, e.g., C1q, C3, C4 and C5 (Muller-Eberhard, 1968), thus interfering with subsequent hemolysis in the system. It is obvious that under such conditions, quantitative studies of 19S and 7S plaque formation could not be carried out.

(C) Specificity of the Plaque Method: Specificity of the plaque method was examined in three ways, i.e., the plating of normal and immune spleen cells with RBC syngeneic or specific with the spleen cell donor; the use of "third party" strains as target RBC donors, and the incorporation of alloantigens in the top agarose layer for inhibition of plaque formation. For the first series of tests, results showed that plaques only appeared when spleen cells were from immune animals and were plated with the specific target RBC. No plaques were observed when immune cells were plated with syngeneic target RBC, or

when the spleen cells were from non-immune animals. This can be interpreted to mean that there was no indication of autoimmunity or that environmental antigens cross-reacting with the alloantigens in question had been presented to these animals, a state of affairs quite at variance with the xenogeneic system (anti-SRBC specifically) in which PFC against heterophile antigens are always present (Jerne et al., 1963; Fuji et al., 1971).

It will be recalled that the H-2 histocompatibility system of the mouse is a complex system composed of at least 19 alleles and 36 serologically defined antigen specificities (Klein and Shreffler, 1971). More than one strain may possess a particular allele and more than one allele may share certain specificities. In other words, cross-reactivity exists among certain alleles. By studying the cross-reactivity of target RBC from properly chosen strains, it was considered possible that the specificity of the plaque technique could be examined further. By expressing the number of plaques as a percentage of those appearing on plates with the specific target RBC, it was observed that the plaque counts obtained with the third party RBC as targets were in general agreement with the degree of expected cross-reactivity, or the number of known specificities shared between third party and immunogen

donor strains. These findings were confirmed by the inhibition of plaque formation experiments in which alloantigens from third party strains, when incorporated in the agarose top layer, were able to partially inhibit plaque formation.

This cross-reactivity was originally described as a serological phenomenon, and has been recognized as due to the public antigens of the H-2 locus (Klein, 1971), and which have been found to be wide-spread among different mouse strains. On the other hand, Brondz in the Gamaleya Institute in Moscow presented evidence which showed that H-2 cross-reactivity does not exist for CMI (Brondz, 1968; Brondz and Snegirova, 1971). However, with refined techniques, Lake et al. obtained results showing cross-reactivity in CMI does exist, and attributed the discrepancy to the insensitivity of the method employed by Brondz (Lake et al., 1973). Klein and Murphy (1973) also showed H-2 cross-reactivity <u>in vivo</u>. Thus, using strains B10 ($H\text{-}2^b$), B10.D2 ($H\text{-}2^d$) and B10.BR ($H\text{-}2^k$) in eighteen different combinations (each combination consisting of a recipient, a lymphoid tissue donor (first donor) and a skin-graft donor (second donor)), they observed that challenging grafts from third party strains were rejected in an accelerated manner.

The results of the specificity experiments also seem to indicate that the D-end of the H-2 locus is a more potent stimulator for antibody production at the cellular level. Similar findings were obtained by Malave et al. (1973). However, it has been reported that in CMI studies, histocompatibility at the K-end evokes a more vigorous immune reactions (Demant, 1970; Rychikova et al., 1971; Demant and Nousa, 1971). Such differences as seen in the plaque assay and those in CMI may be due to some unknown but important characteristics of the two assay systems. In either case, it appears that the region within the H-2 complex at which histoincompatibility occurs is more important for the intensity of the subsequent immune reaction than the number of specificities.

CONCLUSIONS

From the results presented in this thesis, the following conclusions can be made:

(1) A sensitive and versatile hemolytic plaque technique in transplantation immunity studies was developed, using mouse RBC as target (indicator) cells and fresh rabbit serum as the complement source.

(2) Rabbit complement was found to be more effective in developing allo-plaques than guinea pig complement, probably due to the higher cytolytic efficiency of the former with 7S alloantibodies, while guinea pig complement excels rabbit complement in the formation of mouse anti-SRBC plaques.

(3) Allo-plaque formation with the present technique was shown to be highly specific. From the specificity experiments, it was also shown that cross-reactivity among H-2 alleles exists and can readily be predicted from the H-2 chart.

(4) The antigens determined by the D-end of the H-2 locus are more potent stimulators of the PFC response than those determined by the K-end.

(5) Non-H-2 histocompatibility antigens present on the strain A tissue cells contributed a significant fraction of the total immunogenecity recognized by the C57BL/10 recipients.

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