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Some Factors Controlling Immunological Memory Of The Immunoglobulin-m Type

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SOME FACTORS CONTROLLING IMMUNOLOGICAL MEMORY OF THE IgM TYPE

by

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LIST OF ABBREVIATIONS

ALS	-	Anti-Lymphocyte Serum
DEAE Dextran	-	Di-ethyl-amino-ethyl-dextran
EBSS	-	Earle's Balanced Salt
HSA	-	Human Serum Albumin
KLH	-	Keyhole Limpet Haemocyanin
PBS	-	Phosphate Buffered Saline
PFC	-	Plaque-Forming Cells
RES	-	Reticuloendothelial System
SRBC	-	Sheep Erythrocytes

ABSTRACT

Immunological memory for IgM antibodies was studied using various doses of antigen for priming. Localized haemolysis in gel was used for the detection of antibody-forming cells. Lethally irradiated mice were used for the *in vivo* culture of donor cells.

IgM memory was shown to exist for a considerable length of time, by transfer of cells from primed donors to irradiated recipients. IgG antibodies, given passively, inhibited the expression of memory by primed cells. The short life of IgM memory in the intact animals was shown to be artifactual, and most probably due to the induction of an IgG antibody response by the antigen.

Large amounts of antigen for priming resulted in the inhibition of memory. This inhibition could not be removed by washing and transferring the cells to irradiated recipients. It was shown that the cells from donors primed with a large amount of antigen were capable of inhibiting the IgM antibody response of other immunocompetent cells. An increased IgM response in the recipient could be obtained from cells derived from mice primed with a large

amount of antigen if the donors were given passive IgG antibody.

It is concluded that the short life of IgM memory in the intact mice as well as a suppressed IgM response when large doses of antigen are used for priming involve an antibody-mediated immunosuppression.

I. INTRODUCTION

The immune response of an animal to an antigen with which it had previous experience (secondary or anamnestic response) is different from the response produced after a first contact with the antigen (primary response). This difference in the response (memory) can be expressed in several parameters of the response. It is found that in primed animals the onset of the immune response is earlier than in non-primed animals. At the peak of response there is a larger number of cells producing antibody in the primed animals compared to the non-primed, and the total antibody produced at the peak is in greater amount in the primed versus the non-primed animals. The peak of response arrives earlier in the primed animals than in the non-primed animals. The sequence of appearance of different classes of antibodies is altered in the secondary response. Whereas IgG antibodies appear several days after IgM in a primary response, both classes of antibodies appear almost simultaneously in a secondary response. The affinity of

the antibodies produced in a secondary response is greater than the affinity of the antibodies produced in a primary response. The sensitivity of the secondary response to physical and chemical insults is different from that of the primary response.

These changes in the immune response, resulting from previous priming with specific antigen are very important in the understanding of the mechanism of immune response itself, as well as their application to many clinical uses. To understand the mechanism of immune response it is very important to know the effects of various agents and procedures on it. Moreover in the situations where immunosuppression is desired such as in transplantation or in a number of immunological diseases, the factors that augment or suppress the immune response would be of great importance, and hence this study.

The present study of secondary IgM response has been carried out from two basic considerations; its development and regulation. In this thesis we would summarize a large amount of work performed in other laboratories during the last decade and show that both antigen and specific IgG antibodies are important factors in the regulation of IgM memory and its expression as a secondary response. It

would also be shown that the suppression of secondary response by large doses of antigen involves a type of antibody-mediated suppression of immune response.

II. HISTORICAL REVIEW

The capacity of primed animals to give a secondary response, following specific antigenic challenge, has been known for a long time (Glenny and Sudmersen, 1921; Gottlieb et al., 1964; Uhr et al., 1962). The extensive studies of immunological memory, in animals and man, (Barr and Llewellyn-Jones, 1951; Barr and Glenny, 1952) established in a rough quantitative fashion the kinetics of development of immunological memory. These workers showed that, in guinea-pigs, the immunologic memory can be detected in two to four weeks of priming, and lasts for at least one year. In man, following a course of diphtheria and tetanus toxoids, immunologic memory can be present at least twenty years later (Gottlieb et al., 1964).

Earlier studies of 19S (IgM) memory by a number of workers indicated that either memory in IgM system did not exist, or if it did, it had a short life (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964; Bauer et al., 1963). There are however a number of reports where IgM memory has been shown to exist for a considerable length

of time (Nossal et al., 1964; Wigzell, 1966; Cunningham, 1969). A single report (Borel et al., 1964) shows that the use of large doses of SRBC and intradermal route for priming results in a long-lasting IgM priming. Long-lived IgM memory to HSA and BSA in rabbits (Porter, 1966), and to SRBC in mice (Cunningham, 1969), has also been shown, if the challenge was given in the absence of serum antibodies induced as a result of first injection.

A. Extirpation of Organs

1. Effect of Splenectomy

Splenectomy performed at the time of secondary response in rabbits, delayed the secondary response, and reduced the peak titre of antibody (Taliaferro and Taliaferro, 1952). This finding indicates that most of the antibody formation takes place in the spleen.

2. Effect of Thymectomy

In many species removal of the thymus soon after birth results in marked immunological defects affecting both the humoral and cellular immunity (Miller, 1961, 1962, 1962a; Good et al., 1962). The antibody response of neonatally thymectomized animals is variably affected,

depending upon the species of animals, the time of challenge, the nature of antigen used and various other factors. Neonatal thymectomy resulted in severe depression of the immune response to a challenge with tetanus toxoid when pathogen-free Swiss mice were used (Hess et al., 1963; Hess and Stoner, 1966). When coliphage MS-2 was used as antigen, thymectomy reduced and delayed the response to a challenge given twenty-five days after the first injection (Basch, 1966). When primary and secondary responses were induced in the animals of the same age, the secondary response was found to be normal in neonatally thymectomized mice. At this time the primary response of the neonatally thymectomized mice was much reduced and delayed (Sinclair, 1967). Neonatal thymectomy in chickens did not show a consistent effect. No significant effects were produced by thymectomy performed in adult life. In the case of skin homografts in mice (Miller, 1965), and Salmonella H antigens in guinea-pigs (Fichtelius et al., 1961), the thymectomized animals reacted as strongly as the controls to a second challenge.

B. Effect of Physical and Chemical Agents on Secondary Response

1. Effect of X-irradiation

Extensive studies on the effects of x-irradiation on immune response, and immunological memory have been docu-

mented. It was noted that for any immune response, primary or secondary, the result of x-irradiation of an animal is, with a few exceptions, the suppression of immune response. The primary response is more sensitive to x-irradiation than the secondary response (Makinodan et al., 1959). In rats and rabbits, on the other hand, use of large doses of x-rays inhibit both the primary and secondary response, and secondary response is more radio-sensitive in its recovery than the primary response (Simic et al., 1965; Taliaferro and Taliaferro, 1969). These authors have further claimed that while primary response is stimulated by small doses of x-rays, the secondary response is significantly depressed (Taliaferro and Taliaferro, 1969). The secondary response is affected in a permanent way if animals are exposed to x-rays after priming (Porter, 1962; Thorbecke et al., 1964). The differences in the sensitivities of primary and secondary response have been shown to be due to differences in the number of cells responding, and not due to increased resistance of primed cells to x-irradiation (Makinodan et al., 1962).

2. Effect of Reticulo-endothelial System Blockade

Reticulo-endothelial system (RES) and its cellular

constituents play an important role in the development of immune response, although they neither synthesize antibody nor appear to be the precursors of antibody-forming cells (Campbell and Garvey, 1963; Wellensiek and Coons, 1964; Nossal et al., 1964). The phagocytosis of particulate antigens by reticulo-endothelial cells may be an important step in antibody synthesis (Frei et al., 1965; Gallily and Feldman, 1967; Argyris, 1967). Macrophages are thought to process antigen, and this processed antigen stimulates antibody formation by other cells (Fishman and Adler, 1963; Askonas and Rhodes, 1965). A number of studies concerned with the role of RES in immune response, have tried to inhibit normal reticulo-endothelial cell function by radiation, antimetabolites, and steroids (Schwartz and Dameshek, 1963; Gabrielsen and Good, 1967). It has also been shown that immune response can be suppressed, when one uses non-toxic agents, such as mineral oil and carbon suspension for this purpose (Lewis, 1964; Derby and Rogers, 1961). There are conflicting reports concerning the effects of RES blockade on specific antibody production. There are reports that India ink or trypan blue in small doses suppress antibody synthesis in rabbits (Gay and Clark, 1924; Canon et al., 1929). An increased response, on treatment with carbon, or

thorotrast as the blockading agent, is also reported in the literature (Fisher, 1966; Jenkins et al., 1965). Recently Sabet and Friedman (1969, 1969a) obtained marked suppression of 7S memory with colloidal carbon injected twenty-four hours before the primary antigen injection. The peak 7S secondary PFC response was delayed for four days by this treatment. The 19S secondary PFC response was not affected to a great extent and the peak response occurred at the normal time. When carbon was injected twenty-four hours before challenge, the 19S PFC response was suppressed to a greater extent than the 7S PFC response. The time of inoculation of carbon in relation to the antigen was found to be important in producing the suppression.

3. Effect of Anti-Lymphocyte Serum

Anti-lymphocyte serum (ALS) has been shown to suppress cell mediated immune response, and consequently to prolong the life span of second set skin grafts (Levey and Medawar, 1966; Lance, 1968). There is no complete agreement on the mechanism of action of ALS. It is believed that it is mainly active against thymus-derived circulating lymphocytes. No suppression of the secondary response to BSA could be obtained when ALS was used (Lance,

1968; Lance et al., 1968). There are four hypotheses which have most often been proposed to explain the mode of action of anti-lymphocyte serum:

(1) ALS is toxic to lymphocytes, thus reducing the number of lymphocytes available to react to an antigenic challenge. This may be accomplished by the complement-mediated destruction of lymphocytes under the influence of ALS.

(2) Blindfolding of lymphocytes: This states that ALS does not kill lymphoid cells, but coats them so that they cannot recognize foreign antigen.

(3) The competitive antigen theory: The rapid uptake of heterologous IgG, which in itself is immunogenic, induces the lymphoid cells to become active against the immunoglobulin. Therefore they are too preoccupied to react to any other immunogen.

(4) Sterile inactivation theory: The generalized transformation of cells by antibody results in the inactivation of all immunologically non-committed lymphocytes. This results in the loss of immunologic potential.

None of these hypotheses, however, can explain all the experimental data (De Meester, 1969; James, 1967; Levey and Medawar, 1966). ALS can erase immunological memory, and it is considered to be active against readily mobilizable long-lived lymphoid cells (Lance, 1970).

4. Effect of Anti-Theta Serum on Secondary Response

Anti-theta serum has been shown to specifically kill thymus-derived cells, in the presence of complement. Using hapten-protein conjugates in an in vitro system, it was found that anti-theta serum depressed secondary immune response (Raff, 1970). It was shown, moreover, that this depression was due to the effect on antigen-reactive cells of thymus origin and not the antibody-forming cells of bone marrow origin (Raff, 1970).

5. The Effects of Adjuvants on the Secondary Response

Agents that can non-specifically augment the immune response have been used for a long time. These agents have varying effects in different species, and the mechanism of their action is not very well understood. For a long time it was thought that adjuvants increase the immunogenicity of an antigen by acting as depots, from which the antigen is slowly released. This does not seem to be

true, because Bordetella pertussis vaccine enhances immune response of mice even when injected separately from the antigen. Moreover adjuvants frequently induce mitotic response and increase serum immunoglobulin levels, out of proportion to the increase in specific antibody production. Adjuvants can alter the distribution of immune response between cellular and humoral immunity. The activation of lymphoid cells is responsible for the increased responsiveness of the adjuvant treated animals, as can be shown by transfer of lymphoid cells. Simultaneous injection of SRBC and killed cells of B. pertussis prepared the mice for a higher secondary IgG response than the SRBC alone (Finger et al., 1969).

C. Effect of Specific Antibodies on Immune Response

1. Effect of Homologous Antibodies on Secondary Response

It is now well established that homologous IgG antibodies inhibit both primary and secondary IgM and IgG response. IgG antibodies are capable of inhibiting IgG priming (Sinclair, 1969). For a long time it has been known that mixing excess amounts of specific antibody with antigen can suppress the immune response given by the antigen alone (Smith, 1909). Antibody is effective

in suppressing the immune response even if it is given several days after immunization (Uhr and Baumann, 1961, 1961a).

Different classes of antibodies may have differences in their inhibitory capacities. It has been shown that 7S antibodies are more effective than 19S antibodies in inhibiting both 19S and 7S primary response (Finkelstein and Uhr, 1964). It is well known that antibodies are most effective in suppressing the immune response when given before or soon after the antigen, than given at a later time (Moller and Wigzell, 1965; Finkelstein and Uhr, 1964; Horibata and Uhr, 1967). In contrast to this finding, Dixon and his co-workers (1967) found that efficiency of antibody suppression against KLH in rabbits increased markedly if antibody was delayed until Day one after immunization. By labelling the antigen with ^{131}I, these authors found that most of the antigen was removed from circulation within one day of injection. They suggested therefore that the majority of the antigen was catabolized within one day by processes which were irrelevant to antibody production. Uhr and Finkelstein (1967) have shown that sufficient amounts of 7S antibody injected three days after immunization would partially inhibit 19S response at one

week and prevent the development of a 7S response and 7S immunological memory. In general it is more difficult to inhibit priming than the primary response. Doses of antibody which inhibit primary response to diphtheria toxins in rabbits, guinea-pigs and rats, usually do not inhibit sensitization for vigorous secondary response. Large enough doses of antibody can however inhibit priming. This is perhaps due to the fact that much smaller antigen doses are required to induce priming than to induce a primary response (Nossal et al., 1965; Wigzell, 1966). Suppression of secondary response is more difficult to achieve compared to the primary response. This is true when diphtheria toxoid is used in rats and guinea-pigs (Uhr and Baumann, 1961), and when SRBC is used as antigen in rats or mice (Rowley and Fitch, 1964; Morris and Moller, 1968).

Passive administration of IgG antibodies terminated the IgM response (Sahiar and Schwartz, 1964a). This finding indicated that in the time course of a normal response, the termination of IgM response may be affected by IgG antibodies. This idea received further support from the finding that in rabbits treatment with 6-mercaptopurine drastically suppressed the IgG response, and prolonged the IgM response (Sahiar and Schwartz, 1964). Moller and Wig-

zell (1965) studied the suppression of immune response by 19S and 7S antibodies, and concluded that 7S antibodies are far more effective in suppressing an immune response than 19S antibodies. These authors also emphasized, however, that 19S antibodies are several hundred-fold more effective in haemagglutination than 7S antibodies on molecular basis. The difference in the suppressive activity of the two classes of antibodies therefore may be due to the smaller number of 19S molecules being compared to a larger number of 7S molecules. Rowley and Fitch (1964) have shown that antibodies obtained six days after SRBC injection are capable of preventing primary but not a secondary response to SRBC in rats. Pearlman (1966) used 19S and 7S antibodies of equal combining activity and could not find differences in their suppressive activities. Henry and Jerne (1967, 1968) have found that injection of 7S antibodies results in marked suppression of the primary immune response, while 19S antibodies augment the response to SRBC in mice. These authors isolated pure 19S antibodies from sera by sucrose gradient centrifugation or euglobulin precipitation method. They have therefore concluded that other laboratories which reported suppression of immune response by 19S antibodies may have obtained these results

with 19S contaminated with small amounts of 7S antibodies.

Bystryn and co-workers (1971) have developed a model for the regulation of immune response by specific antibodies. According to this model, the regulation of immune response is mediated through the formation of antigen-antibody complexes of various ratios. In this model the antibody important in regulating the immune response would be the one which can enter extravascular space and reach the immunogen containing compartment. Since IgG penetrates this space more easily (Waldmann and Strober, 1969), their model would ascribe the regulatory role to this class of antibody.

2. Suppression of Immune Response by Antibody Fragments

Antibody may influence the immune response by combining with the antigen and making it unavailable to stimulate the antigen-sensitive cell. It could also inhibit the antibody-forming cell or its precursors directly. This may involve transmission of a negative signal to the precursors of antibody-forming cells, through the Fc fragment of antibody (Sinclair and Chan, 1971). It is known that Fc is important in many of the biological functions of antibody, such as complement fixation (Tranta and Franklin, 1961), placental passage (Brambell et al., 1960), cytophilia (Berken

and Benacerraf, 1966; Uhr, 1965) and reabsorption from the renal tubules. It is therefore important to know the effects of the removal of Fc fragment of antibody on its immunosuppressive activity. It is known that controlled digestion of IgG by pepsin results in the removal of the Fc fragment from the antibody. Furthermore this does not significantly affect the ability of the remaining $F(ab')_2$ antibody to combine with the antigen. The removal of Fc fragment from the antibody, however, results in rapid excretion of the remaining $F(ab')_2$ fragment from the body (Spiegelberg and Weigle, 1965).

Studies where comparisons of the suppressive activity of intact 7S antibody and its $F(ab')_2$ have been made, have yielded conflicting results. Tao and Uhr (1966), Uhr (1968) and Greenbury and Moore (1968) have shown that $F(ab')_2$ is effective in suppressing the immune response. On the other hand, there are reports (Sinclair, 1969; Sinclair et al., 1968, 1970), which show that $F(ab')_2$ is much less effective in suppressing the immune response. When a single injection of antibody was used, $F(ab')_2$ was found to possess one-hundredth to one-thousandth the activity of whole antibody in suppressing IgG memory (Sinclair, 1969). To compensate for the more rapid excretion of $F(ab')_2$,

multiple injections of $F(ab')_2$ were used. It was found that the $F(ab')_2$ was still significantly deficient in suppressing the immune response (Sinclair et al., 1970). Using antibody treatment in vitro, where excretion could be ruled out, it has been found that $F(ab')_2$ is less effective than intact IgG in suppressing an immune response (Lees and Sinclair, 1972).

D. The Nature of Memory

The question, what constitutes immunological memory has not been satisfactorily answered. A considerable amount of work has been done to answer this question and some ideas can be formed at this point. It is known that increased responsiveness of the animal on priming results from an increased number of cells. These cells can be functionally distinguished from the virgin antigen-sensitive cells. These cells are known to be long-lived at least in case of IgG memory, and do not divide in the animal without further contact with the antigen (Gowans et al., 1962; Gowans and McGregor, 1963). The memory cells are morphologically similar to small lymphocytes (Gowans and Uhr, 1966). The drainage of lymphocytes from the thoracic duct of rat for five days does not completely deplete the donor of secondary

reactivity. This indicates that some of the memory cells may not be easily mobilizable, and may be localized in organs in a more or less sessile form (McGregor and Gowans, 1963). Other candidates for memory cells may be macrophages, which may carry information in the form of an RNA-antigen complex (Fishman and Adler, 1963). It has been suggested that mature plasma cells which are known to have a long life span may serve as the carriers of immunological memory (Makinodan and Albright, 1964). There is, however, a large amount of evidence which points towards small lymphocytes being the carriers of immunological memory (Bosman and Feldman, 1968). These authors have found that there is a sudden decrease of mature lymphocytes during the anamnestic response. The evidence against plasma cell being the carrier of immunological memory was provided by Wakefield and Thorbecke (1968, 1968a). They showed that while both red and white pulp of spleen contained antibody-forming cells after an initial antigen injection, the cells which responded by proliferation to an antigen challenge were present only in the white pulp (Wakefield and Thorbecke, 1968a). Further it was shown that antibody-forming cells from the centre of plaque were unable to transfer immunological memory in irradiated hosts (Cunningham, 1969).

Moreover, the transfer of memory could be accomplished by transferring non-plaque-forming cells from the same preparation.

Recent work from many laboratories has shown that there is a synergistic interaction between bone marrow and thymus-derived cells in the induction of immune response to certain antigens (Claman et al., 1966, 1966a; Davies et al, 1967; Mitchell and Miller, 1968; Taylor, 1969). Thymectomy at birth, adult thymectomy and total body irradiation followed by bone marrow protection, is associated with a diminished capacity to give certain immune responses. In lethally irradiated mice there is very little response to SRBC, when only thymus cells are transferred (Davies et al., 1967). By using anti-allotype sera and chromosome markers, it has been shown that antibody-forming cells are derived from bone marrow (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Nossal et al., 1968). This synergistic interaction between two cell types may be accomplished by several mechanisms. The simplest mechanism would be by concentrating antigen, or presenting it to the appropriate cell, in such a fashion that it could stimulate the immune response Taylor, 1969). The question then is, which of the two cell types is the memory cell? Does one of the two cell types

carry memory or both of them do? Does interaction between the two cell types occur in the generation of memory? Using allotype specificity controls (Jacobson et al., 1970), in an adoptive transfer system, where virgin thymus and bone marrow cells were derived from allotype congenic strains, it was shown that all PFC detected in the recipient spleens carried marrow allotype. When the same system was used for the study of possible interaction between memory cells derived from primed spleens, and normal bone marrow cells, no such evidence for cooperation was found. This suggests that cooperation is only necessary in priming. Miller (1970) using thymectomized irradiated recipients, or neonatally thymectomized recipients reconstituted with marked thymus cells, has shown that immunological memory resides in thymus-derived cells. Moller (cited in Celada, 1971), using anti-theta serum in vitro, to destroy thymus-derived cells from the primed spleen cells, has obtained evidence that this treatment inhibits the secondary immune response.

The problem then is whether memory cells belong to the progeny of thymus- or bone marrow-derived cells, or to both; is there any qualitative difference between a virgin antigen-sensitive cell and a memory cell. There is some

evidence that memory cell is different from antigen-sensitive cell in some of its characteristics. It has been shown (Rowley and Fitch, 1964) that secondary response is much more difficult to suppress by antibody than a primary response. Tolerance induction is extremely difficult in primed animals compared to non-primed animals. Memory cells are in a committed state for differentiation (Perkins and Makinodan, 1964). Byfield and Sercarz (1969) have shown that in their system memory develops within twenty-four hours of the primary antigen. Moreover, they have shown that while inhibitors of DNA synthesis like methotrexate and hydroxyurea do not have any effect on the development of memory within twenty-four hours of primary antigen, they do inhibit the development of memory after twenty-four hours. This indicates that the memory which develops within twenty-four hours of primary antigen is due to a change in the responsiveness of the cells and not due to an increase in the number of cells. Therefore this indicates a qualitative difference between cells responding to a first antigen contact, compared to those which respond to the second antigen contact.

E. Relationship of Secondary Response to Antigen Dose

Antigen plays an important part in various facets of

memory. The dose of antigen given for priming determines the development of memory, the rate of its development, the amount of memory which would develop, and the expression of memory in quantitative as well as qualitative terms. This means that the priming dose determines whether memory would be expressed in terms of large numbers of responding cells on antigenic challenge, and what class of antibody would be produced. The antigen dose required for the establishment of significant memory is smaller than that required for the induction of primary response (Nossel et al., 1965). The optimum priming dose of antigen to obtain a maximum IgM secondary response upon antigenic challenge is smaller than that needed for maximum IgG response (Valentova et al., 1966, 1967). The peak of priming for IgM response is observed in a considerably shorter time compared to the peak of priming for IgG response (Valentova et al., 1966). Recently, it has been shown (Wigzell, 1966) that small amounts of antigen used for priming result in a significant amount of IgM priming. This IgM priming, however, does not last very long and seems to be of transient nature. Use of adoptive transfer of cells into irradiated recipients has established that these cells show secondary responsiveness upon transfer into irradiated

recipients (Cunningham, 1969). It therefore seems that, in the intact animal, memory is present but cannot be expressed upon challenge due to some functional block. Large amounts of antigen given for priming have been shown to result in a primary IgM response. Lymph node fragments from rabbits given large doses of antigen for priming were shown to give a poor response upon secondary antigenic challenge. This response lasted for a short time, after which the lymph node fragments became unresponsive for some time (Byers and Sercarz, 1968).

Two major states of antigen-induced specific unresponsiveness have been documented in literature, immunological tolerance (Owen, 1945; Billingham et al., 1953; Hasek et al., 1961; Smith, 1961) and immune exhaustion (Felton, 1942; Sterzl and Silverstein, 1967; Byers and Sercarz, 1968). The two states may or may not involve different mechanisms. There is, however, no clear evidence yet to indicate that the two phenomena are different. According to X-Y-Z scheme of Sercarz and Coons (1962), tolerance is a complete and specific inhibition of immune responsiveness of X antigen-sensitive or Y memory cell to a particular antigen. Exhaustion is the productive expenditure of a particular compartment of immunocytes (Byers

and Sercarz, 1968). The essential difference between tolerance and exhaustion is that while in tolerance the animals or cells go into a state of unresponsiveness without producing large amounts of antibody, in exhaustion there is considerable antibody production before the animals or cells become unresponsive. Moreover, the term "tolerance" was initially used to define immunological unresponsiveness towards living foreign antigens such as viruses or lymphoid cells. According to the hypothesis of Byers and Sercarz (1968), four possibilities of specific unresponsiveness exist:

(1) Tolerance of X-cells (2) Exhaustion of X-cells

(3) Tolerance of Y-cells (4) Exhaustion of Y-cells

Recent studies on the mechanism of antibody induced immunosuppression have indicated that induction of tolerance may involve a mechanism similar to antibody induced immunosuppression (Bretscher and Cohn, 1968, 1970; Sinclair and Chan, 1971a; Feldmann and Diener, 1971).

The use of large amounts of antigen for priming results in a depressed immune response on antigenic challenge in vivo (Hanna and Peters, 1971) and in vitro (Sercarz and Coons, 1962; Byers and Sercarz, 1968). This depression of immune response has been explained on the basis of exhaus-

tive differentiation of antigen-sensitive X-cells, upon repeated antigenic contacts, to irreversibly matured antibody-forming cells (Byers and Sercarz, 1968; Hanna and Peters, 1971). This maturation of antigen-sensitive X-cells to antibody-forming Z-cells, with consequent depletion of memory or Y-cell compartment can be prevented by passive transfer of specific antibody (Hanna et al., 1969). The recovery of immune responsiveness with antibody has been explained on the basis of neutralization of excess antigen, which might fire off the differentiation of Y-cells to antibody-forming Z-cells (Hanna et al., 1969). There are other possibilities which could give similar results, for instance carry-over antibody being transferred with the donor cells, or antigen antibody complexes accompanying the cell transfer could suppress the immune response. These possibilities have not been completely ruled out.

III. MATERIALS AND METHODS

A. Mice

Swiss mice originally obtained from Carworth Farms, New City, New Jersey, U.S.A. and inbred by strict brother x sister mating in this laboratory for over fifty generations were used for all experiments. The mice were maintained in groups of six in disposable plastic cages (Disposable Econo Cages, Maryland Plastics Inc., New York, N.Y., U.S.A.). The mice were provided with a bedding of Ab-Sorb-Dri (Michael Woods Products Inc., Garfield, New Jersey, U.S.A.). They were given water and Purina Laboratory Chow ad libitum. The water bottles were autoclaved weekly and filled with fresh water. Each experimental group usually contained from four to six mice.

B. Antigen and Immunization

Sheep erythrocytes (SRBC) in Alsever's solution were obtained from Grand Island Biological Co. (GIBCO), Grand Island, N.Y., U.S.A. or from SYLAB (Box 313, Sarnia, Ontario, Canada). The cells were washed three times in

isotonic saline (0.9% NaCl) and resuspended in saline to obtain the desired concentration. The cells were always counted in a haemocytometer ('Bright Line', American Optical Co., Instrument Division, Buffalo, New York) before intravenous injection for priming. All injections were made through the lateral tail veins of the mice.

C. Anti-Sheep Erythrocyte Antibody for Passive Immunization

Mice were given two injections of 0.1 ml of 10% suspension of SRBC at a one month interval, and the serum was collected about one month after the second injection. The mice could be bled repeatedly for several months and maintained high titres of antibodies. This serum did not contain any detectable 19S antibody, and was considered to be the IgG antibody-containing serum.

D. Preparation of $F(ab')_2$ Antibody from IgG Antibody

The serum, obtained one month after the second of two injections of SRBC to mice, was used for the preparation of $F(ab')_2$ from IgG antibody. Following three ammonium sulphate precipitations (40% saturation), digestion was carried out in a 0.07 M sodium acetate and 0.05 M sodium chloride buffer pH 4.0 at $37^{o}C$ for 18 hours in the presence of 1 mg

of pepsin (2650 units/mg, twice crystallized and lyophilized, obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.) per 100 mg of protein (Nisonoff et al., 1960). Following pepsin digestion or exposure to acetate buffer, antibody samples were dialysed at 4°C against phosphate-buffered saline (PBS) (0.8% NaCl, 0.1 M phosphate, pH 7.8). Pepsin-digestion decreased the haemolysin titres by up to 5 $\log_2$ units without altering the haemagglutinin titres. The 5S $F(ab')_2$ and 7S (intact IgG) anti-sheep erythrocyte antibodies were separated on a 5-20% sucrose gradient in PBS. To resolve the 5S and 7S antibodies into two peaks, the gradients were centrifuged in SW-40 swinging-bucket titanium rotor, which accommodates 9/16 x 3-3/4 inch nitrocellulose tubes. The gradients were centrifuged at 40,000 rpm for 40 hours at 5°C in the Beckman L2-65B ultracentrifuge. Fractions were collected by puncturing the centrifuge tube at the bottom with a No. 18 lumbar puncture needle and each fraction was tested for haemolysin and haemagglutinin activity. The fractions from each peak were then pooled together for use.

E. Preparation of Rabbit Anti-Mouse Immunoglobulin Serum

Mouse serum containing anti-sheep erythrocyte IgG

antibodies was heated at 56°C for 30 minutes to inactivate the complement activity. The serum was cooled and mixed with an equal volume of 10% SRBC (washed 3times in saline). The mixture was allowed to stand at room temperature for two hours. The SRBC were spun down in a Sorvall general laboratory centrifuge GLC-1 (Sorvall, New Town, Connecticut) at 2000 rpm for 10 minutes. The pellet was reconstituted to 10% suspension in saline and again treated with equal volume of the serum. A further one hour was allowed for the reaction, and SRBC were washed three times in large volumes of saline, to remove any free antibodies, and reconstituted to a 10% suspension. Three to four injections of 1.5 ml of this sheep erythrocyte preparation were given intravenously at 15-30 days intervals to the rabbits weighing 3-4 kg. The rabbits were bled at least twenty days after the last injection. The serum obtained from the bleedings was heated at 56°C for 30 minutes to inactivate endogenous complement. The cooled serum was absorbed several times with washed SRBC until no anti-SRBC activity could be detected by haemagglutination technique. The absorbed serum was stored at -20° until used. Usually the serum gave maximal development of IgG PFC at a dilution of 1:50 to 1:100.

F. Irradiation of Mice

Mice that were irradiated received a lethal dose of total body irradiation (800-850 rad). A Gammacell 20 small animal irradiator (Cunningham, Bruce and Webb, 1965) was used for this purpose. The mice received the radiation at an absorbed dose rate of 7.2×10^3 rad of total body γ-irradiation per hour from two 1000 curie sources of ^{137}Cs. During irradiation the mice were placed in a perspex container which fits in the Gammacell. Air was constantly supplied to the drawer of the irradiation unit and the mouse container in it.

G. Preparation of Cells for Transfer to Recipients

The spleens were removed from the donor mice, and homogenized in ice-cold Medium 199 (GIBCO, Grand Island, N.Y.) in a homogenizer with a loosely fitting piston. The larger chunks were allowed to settle at the bottom of the tube for about two minutes and the single cell suspension from the top was removed with a Pasteur pipette. The cells were washed two to three times in large volumes of ice-cold Medium 199 at 1000 rpm in Sorvall GLC-1 centrifuge for 10 minutes. The cells were always counted in a haemocytometer before injection to the recipients. The

counts were made in 2% acetic acid as diluent as it lysed erythrocytes. The antigen was usually mixed with the cells before injection.

H. Haemolytic Plaque-Forming Cell Assays

Spleen cell suspensions for assay were prepared by gentle homogenization with a loose fitting tissue homogenizer. The IgM (Direct) and IgG (Indirect) plaques were detected by the techniques of Jerne et al. (1963) and of Dresser and Wortis (1965) respectively. In brief, a dispersed spleen cell suspension was prepared and 0.1 ml aliquots were added to tubes containing 3 ml warm (45°C), melted, 0.7% purified agar (Difco Laboratories, Detroit, Michigan) in Earle's Balanced Salt Solution (EBSS, Gibco Laboratories), to which 0.1 ml of a 20% suspension of washed SRBC and 2% DEAE Dextran had been added. This mixture was quickly poured into a previously prepared Petri plate 100 x 15 mm square with a 13 mm grid (Falcon Plastics, Los Angeles, California, U.S.A.), containing a base layer of solidified 1.4% Purified agar in EBSS. The plates were incubated for one to two hours at 37°C and then treated with 3 ml of 1:15 dilution of guinea-pig complement (Grand Island Biological Co., Grand Island, New York, U.S.A.) when direct PFC's were being assayed. The plates

were incubated for one hour at 37°C. At the end of incubation, complement was poured off and direct plaques were counted against a standard light source. If indirect PFC assay was carried out, the plates after the first incubation were treated with 2 ml of an appropriate dilution of rabbit anti-mouse γ-globulin antibody and incubated for one hour. The rabbit anti-mouse γ-globulin antibody was poured off at the end of incubation and complement was added to the plates. From this step onwards the same procedure as for the direct plaques was followed. The number of direct PFC obtained was subtracted from the PFC obtained in a set of identical plates which were treated for indirect plaques. This gave the true value of IgG plaque forming cells.

Initially all spleens were counted and the results expressed as plaques per million spleen cells. There was little variance in the spleen cell counts so that subsequently results were expressed as plaques per spleen. Spleens yielded counts of the order of 2×10^8 nucleated cells per animal.

I. Measurement of Serum Haemolysin Activity

Blood was obtained at various times after immunization from the retro-orbital sinus, and diluted two parts blood to one part saline to avoid gel formation in the serum. The serum was collected following centrifugation and endogenous complement inactivated by incubating the serum at

56^{o} for 30 minutes. Serum samples were serially diluted 1:1 with 0.9% saline in Microtiter plates (Cooke Engineering Co., Arlington, Virginia, U.S.A.), 0.025 ml of 5% guinea-pig complement (Gibco Laboratories) was added, and the serum and complement incubated for 30 minutes at 37^{o}. The pre-incubation of serum and complement lowered the incidence of titrations which were negative at low dilutions but became positive at higher dilutions. Washed sheep erythrocytes (0.05 ml of a 0.5% suspension) were added and the complete mixture incubated for two hours at 37^{o} and then read, the end point being roughly half haemolysis on visual inspection. The plates were stored overnight at room temperature and read again the following morning. Titres were usually one $\log_2$ unit higher on the second reading. All titres are expressed as the $\log_2$ of the dilution on first reading.

IV. EXPERIMENTAL RESULTS

A. EXPERIMENTS IN INTACT ANIMALS

1. A TIME COURSE OF PRIMARY AND SECONDARY IgM RESPONSE

Introduction

To study IgM memory it is important to select a point in the time course of the immune response where the response is at or nearly at its peak, as one would like to show a maximum difference in the animals on antigenic challenge before and after priming. Moreover, since antibody producing cells have a short life span and IgM antibody has a short half life, assay of immune response a long time after the peak could easily lead to wrong conclusions. It is for this reason that the time course was studied to select a proper point at which the immune response would be assayed after various treatments.

Procedure

Mice of the same sex and about the same age were selected for each experiment. For priming, mice were given

a single intravenous injection of 5×10^5 sheep erythrocytes (SRBC) in 0.2 ml saline. Six days after this injection, groups of mice were challenged with 0.2 ml of 10% (4×10^8) SRBC washed three times in saline. Various times after the challenge mice from primed and non-primed groups were bled and killed by cervical dislocation. The spleens of the sacrificed animals were removed and suspended in ice cold Medium 199. Cell suspensions from the spleens were prepared by gentle homogenization and assayed for plaque-forming cells (PFC).

Results

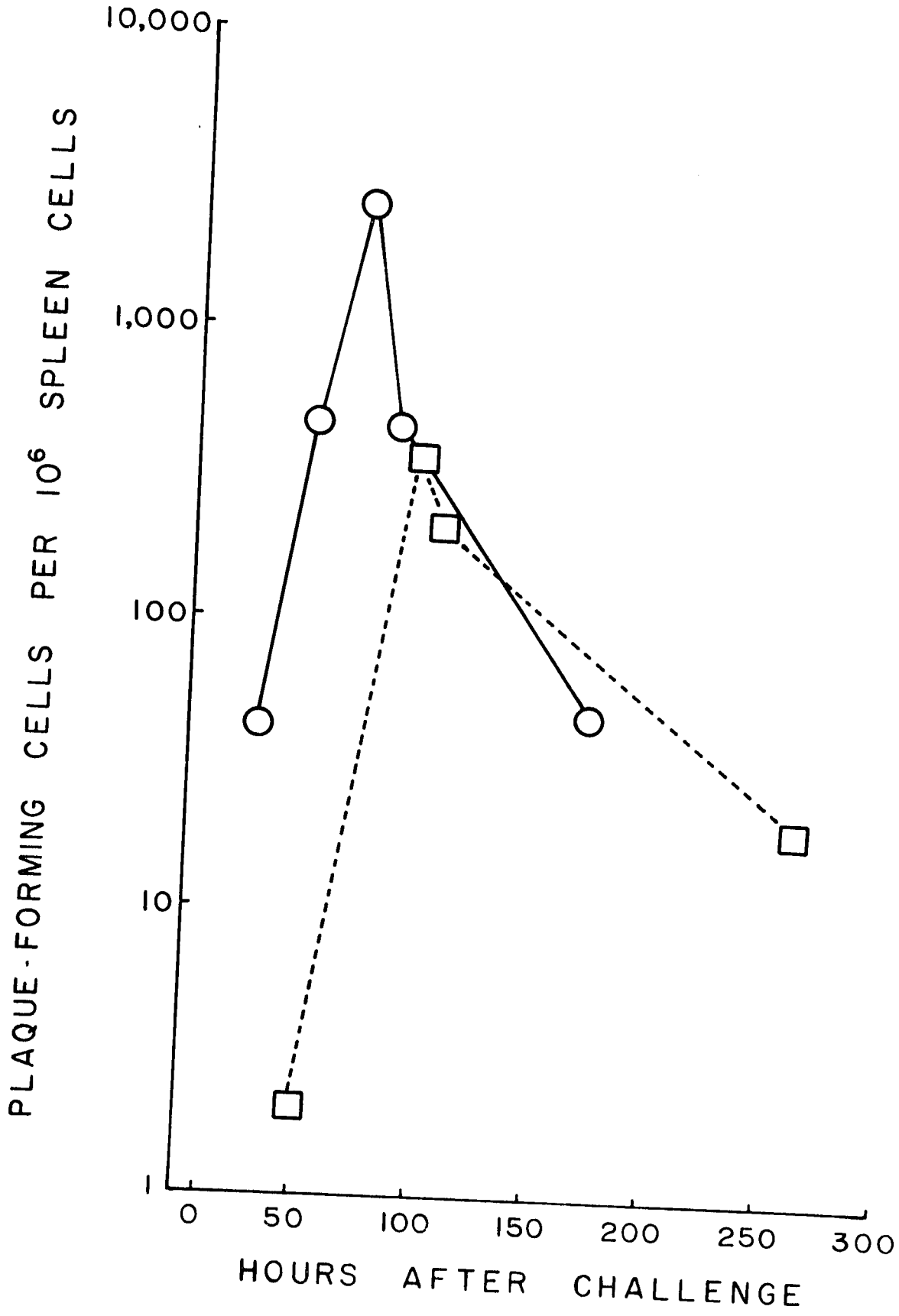
As can be seen in Fig. 1, spleens from primed mice had large numbers of PFC 30 hours after the challenge injection. The number of PFC in these spleens rapidly increased and reached a peak about 70 hours after the challenge injection. Subsequent to this peak, there was a rapid decline in the number of PFC. About 100 hours after the challenge, primed and non-primed animals had the same number of PFC in their spleens.

In contrast to the secondary response, the spleens from mice, which had not previously received a priming antigen injection of SRBC, showed a low number of PFC 50 hours

FIGURE 1

A TIME-COURSE STUDY OF PRIMARY AND SECONDARY IgM RESPONSES IN INTACT ANIMALS

Direct (19S) PFC response of normal (□) and primed (○) Swiss mice were measured at various times after a challenge with 4×10^8 SRBC. The primed mice were injected with 5×10^5 SRBC six days before challenge. Each point represents the arithmetic mean of PFC per million spleen cells of three to four mice. The number of plaque-forming cells, calculated per million spleen cells or per whole spleen, gave similar results.


10,000
1,000
100
10
1
PLAQUE-FORMING CELLS PER 10^6 SPLEEN CELLS
0
50
100
150
200
250
300
HOURS AFTER CHALLENGE

after the challenge. The numbers of PFC rapidly increased and reached a peak about 100 hours after the antigen. Beyond the peak the immune response of non-primed mice also declined rapidly. It is of interest to note that, at the peak primary response, the secondary response had already declined so much that there was no difference between primary and secondary response. At the time when secondary response reached its peak, it was about 100 fold higher than the primary response. Therefore 72 hours (Day three) of the immune response was selected for the study of IgM memory in further experiments. This time course of primary and secondary immune response is in general agreement with the reports from other laboratories.

2. A TIME COURSE OF IgM IMMUNOLOGICAL PRIMING

Introduction

Since the discovery of various classes of immunoglobulins and their further characterization, marked differences have been observed in their properties. This has stimulated interest, and several attempts have been made to study the properties of individual immunoglobulin classes. It has been found that immunological memory of the IgG class of antibodies lasts for a long time after

the priming antigen injection. In the case of IgM priming, however, differences have been reported by various workers. Some of the workers have failed to demonstrate any IgM priming while others have been successful in obtaining priming, but have found that it lasts for a short time only. In order to further clarify this point, we have studied the time course of priming.

Procedure

To determine the time course of IgM memory, mice were injected intravenously once with 5×10^5 SRBC in a 0.2 ml volume. Varying times after this initial injection, groups of mice were challenged with 0.2 ml of a 10% suspension of SRBC (4×10^8 SRBC). At the time of challenge of previously injected mice, a group of normal mice was also given the latter dose of antigen and served as a control primary response. The contribution to the response by the first antigen alone was evaluated by assaying some mice which received the first antigen injection but did not receive a second exposure to the antigen. Three days after the primed mice received the challenge, all groups of mice were bled, killed and their spleens assayed for PFC response. The sera obtained from these mice before sacrifice were titrated

for haemolysin activity.

Results

Fig. 2 shows the immune response assayed as PFC and Fig. 3 shows the haemolysin activity in the sera of the mice. It can be seen that the priming antigen injection alone did not increase the response of mice to a very large degree at any time during the studies. There was some increase in the response over the background response of untreated mice more than a week after the priming dose. However, the contribution of this response towards the secondary response was never more than 5% of the total PFC. It is also evident that priming injection increased the response to a challenge to over ten times at the peak of priming. Priming was definitely established within four days after the priming antigen injection and increased during the following days, reaching a peak at about one week. Subsequent to the peak of priming, there was a rapid decline in the memory. Within ten days of the peak, only one-fifth the value of the peak response could be obtained upon antigenic challenge. The initial rapid decline in memory was followed by a slower decline in the immunological memory. These results are in general agreement with

the results reported by Wigzell (1966) and Cunningham (1969). These experiments were repeated a number of times with similar results. In another experiment (Fig. 4), IgM memory was studied up to one hundred and twenty-five days. The results are expressed as index of priming which is the number of PFC given by primed animals divided by the number of PFC given by non-primed animals. It is seen that the results are quite similar to the other experiments (Fig. 2). However, in this experiment as well as in some of the other experiments, a second increase in the responsiveness is observed between twenty and forty days. This increase, however, did not occur in all of the experiments where memory was followed beyond twenty days.

Discussion

The increased response given by primed animals upon challenge, is not due to a larger amount of antigen received by them in two injections, compared to the primary controls which received only the challenge dose. It is clear from the results that the sum of responses given by either injection alone is far below the response when the two injections were used in succession. Moreover, if it had been an additive effect of the antigen dose, one would

FIGURE 2

A STUDY OF THE TIME-COURSE OF IgM IMMUNOLOGICAL MEMORY

Direct PFC responses of normal (□) and primed (○) Swiss mice were measured three days after a challenge with 4×10^8 SRBC. The primed mice were injected with 5×10^5 SRBC various times before challenge. The background response was assessed in normal mice which did not receive antigen (normal background) (⬡). The response of mice primed for various times but not given the challenge (▽) was assessed at the same time as that of primed and challenged mice. Each point represents the arithmetic mean of PFC per spleen of five to six mice and the vertical lines represent one standard deviation of the reported mean. The number of plaque-forming cells, calculated per million spleen cells or per whole spleen, gave similar results.

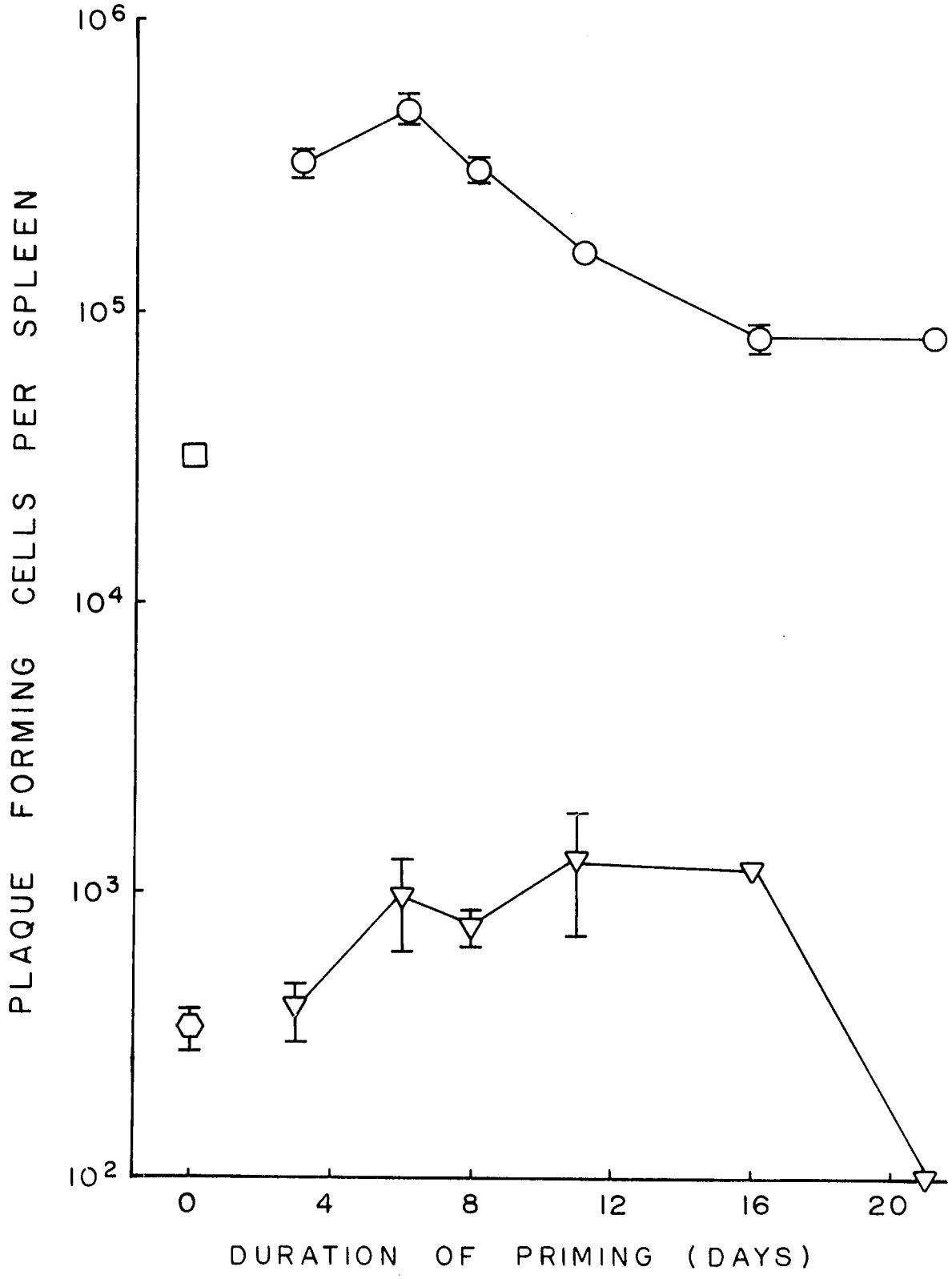

PLAQUE FORMING CELLS PER SPLEEN
10^6
10^5
10^4
10^3
10^2
0
4
8
12
16
20
DURATION OF PRIMING (DAYS)

FIGURE 3

A TIME-COURSE OF IMMUNOLOGICAL PRIMING STUDIED AS TOTAL HAEMOLYSIN RESPONSE OF INTACT MICE

Total haemolysin responses of normal (□) and primed (○) Swiss mice were measured three days after a challenge with 4×10^8 SRBC. The primed mice received 5×10^5 SRBC various times before challenge. The background response was assessed in normal mice which were not given antigen (◇). The response to priming antigen dose was measured in mice primed for various times but not given the challenge (▽), at the same time as that of primed and challenged mice. Each point represents the arithmetic mean of the haemolysin titres ($\log_2$) of five to six mice and the vertical line represents one standard deviation of the reported mean.

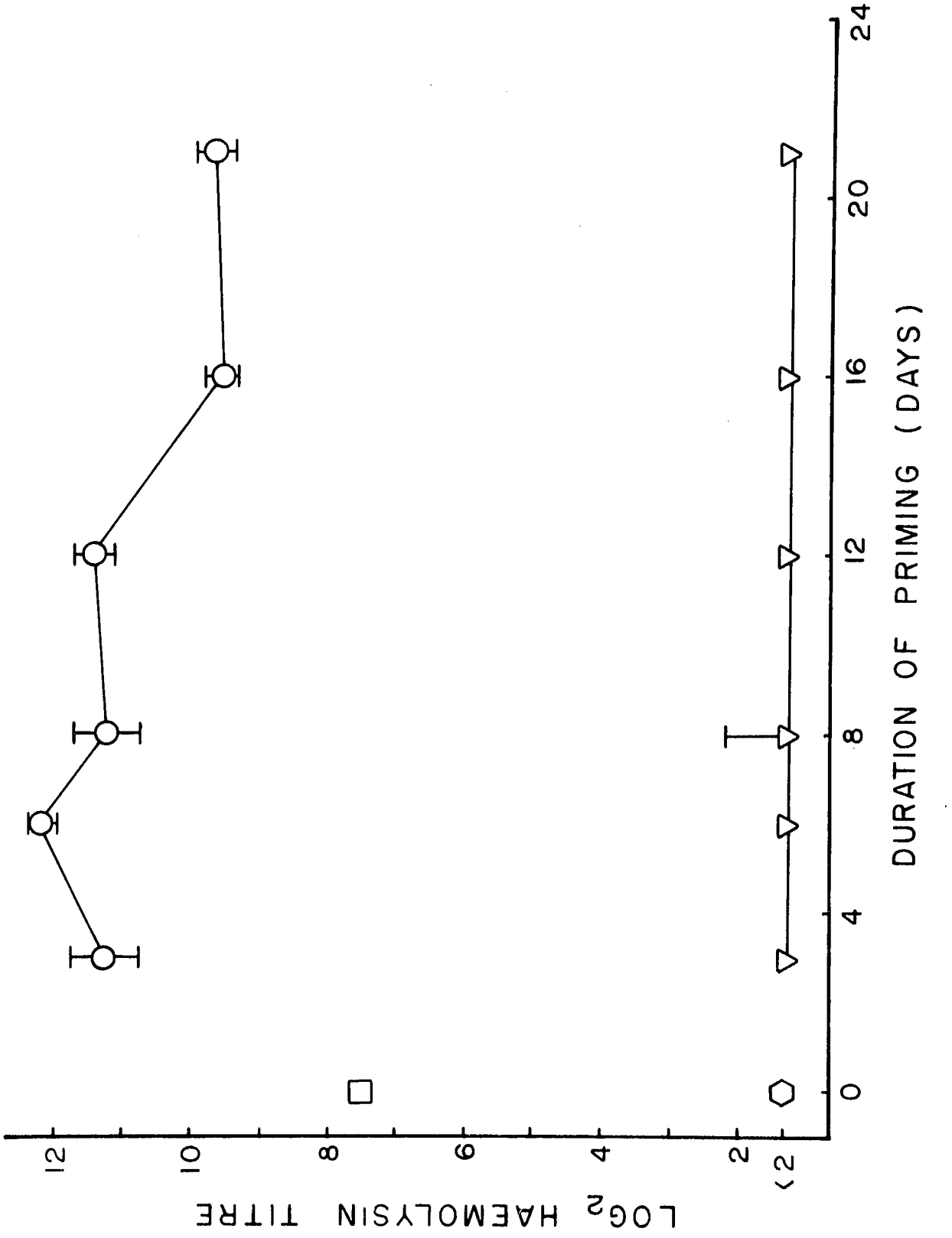
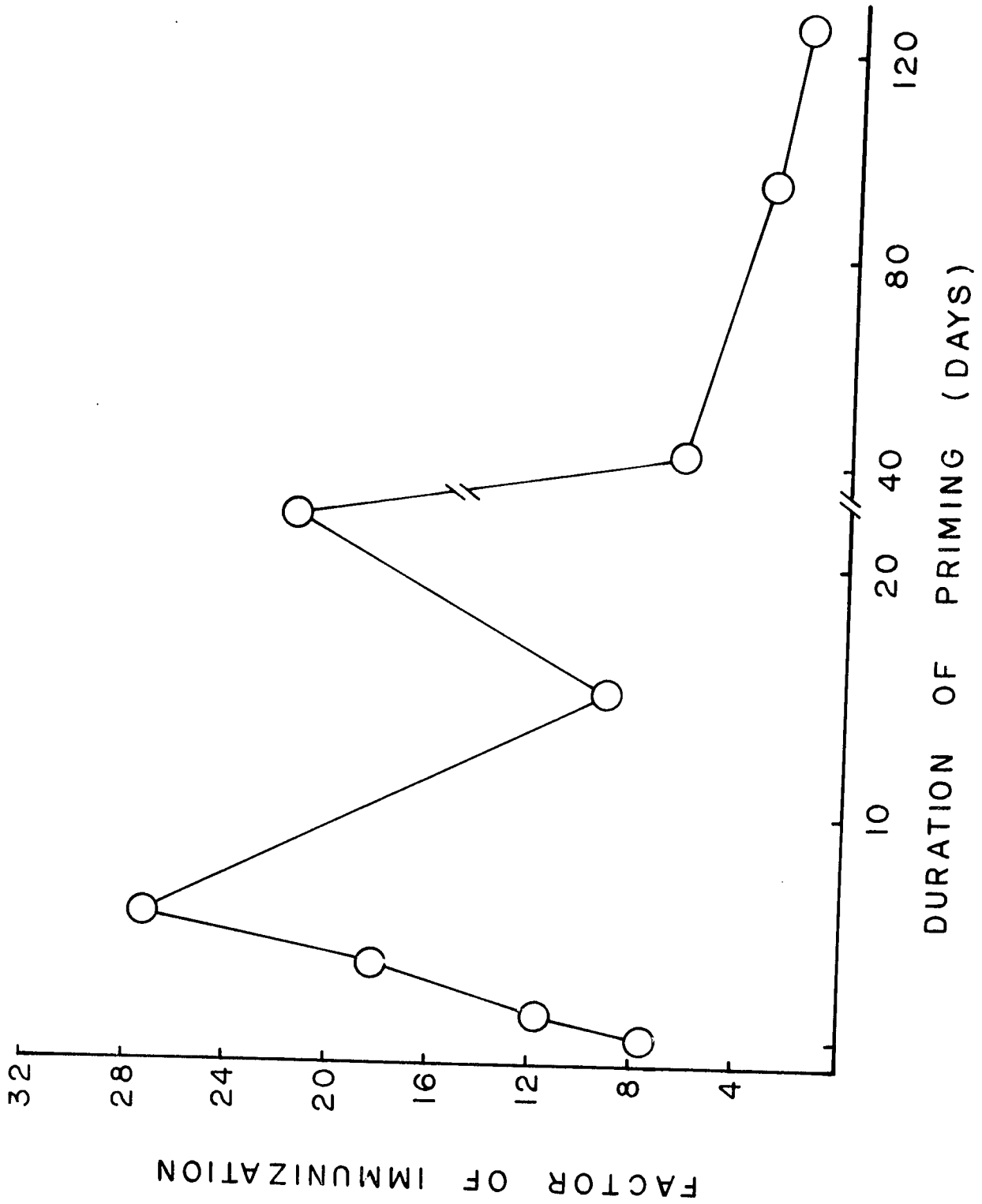

LOG2 HAEMOLYSIN TITRE
12
10
8
6
4
2
<2
0
4
8
12
16
20
24
DURATION OF PRIMING (DAYS)

FIGURE 4

A TIME-COURSE OF IgM IMMUNOLOGICAL PRIMING STUDIED AS THE FACTOR OF IMMUNIZATION

The direct PFC response of Swiss mice primed for various times with 5×10^5 SRBC was measured three days after a challenge with 4×10^8 SRBC. The factor of immunization (○) was calculated by dividing this by the PFC response of normal mice three days after the challenge. Each point represents the factor of immunization calculated from the response of three to eight mice.


FACTOR OF IMMUNIZATION
32
28
24
20
16
12
8
4
10
20
40
80
120
DURATION OF PRIMING (DAYS)

have expected to obtain the maximum increase in response when the two injections were given close together, since it is at this time that the largest amount of antigen would be present in the body of the animal. At later intervals, a large proportion of the antigen, given in the first injection, would have been catabolized and removed from the body.

3. SUPPRESSION OF IgM SECONDARY RESPONSE BY IgG ANTIBODIES GIVEN BEFORE THE ESTABLISHMENT OF PRIMING

Introduction

It is well established that IgG antibodies transferred passively into animals inhibit primary IgG and IgM responses, and also IgG priming. It has also been reported that IgG antibodies inhibit IgG and IgM secondary response. It is possible that the decline in IgM priming within one month of the first antigen injection is due to the production of IgG antibodies. The decline of IgM secondary response starts about ten days following priming, and by twenty days there is a considerable decline in the secondary response upon challenge. This is the time at which IgG response has been shown to increase when larger doses

of antigen are used. To test the effects of passively transferred IgG antibodies on the secondary IgM response, experiments were performed in the following manner.

Procedure

Mice were primed with a single intravenous injection of 1×10^6 SRBC. One day after the priming injection, various quantities of IgG antibodies were injected to different groups of mice. Thirty-six days were allowed after the antibody injection for the passively transferred antibodies to decay. All groups of mice were then challenged with 4×10^8 SRBC. At the same time a group of normal mice received the challenge dose to serve as a control primary response. A group of mice which had received the priming dose of antigen thirty-seven days before, but did not receive antibody, was also challenged at this time. Three days after the challenge, all mice were bled, and their spleens assayed for PFC.

Results

In Figs. 5 and 6, it can be seen that IgG antibody has a marked inhibitory effect on IgM secondary response. The inhibition is greater when a large dose of antibody is used, and becomes proportionately less when the amount

of antibody injected is reduced. Assuming the half-life of IgG antibodies in the mouse to be five days, there should be less than 1/100th the amount of antibodies still remaining in the mice, at the time when they were challenged. The smallest amount of antibody, which was effective in suppressing the immune response, was less than 1/30th of a ml. It is obvious that a very small amount of antibody is sufficient to suppress IgM response. The larger amounts of IgG antibody inhibited the IgM secondary response to the extent that it did not appear different from the primary response.

Discussion

Even though the challenge was given at Day thirty-seven, there was still significant priming to study the effects of IgG antibody on priming. It seems that IgG antibodies inhibit priming. This, however, can not be said with certainty, because inhibition of secondary response in any other way would give similar results. For instance, if priming had been established but memory cells were inhibited from responding, the results would be similar. Therefore on the basis of this experiment, we can say that IgG antibodies suppress IgM secondary response, but we cannot dis-

FIGURE 5

SUPPRESSION OF IgM SECONDARY RESPONSE BY IgG ANTIBODIES

The direct PFC responses of normal (□), primed (○) and primed antibody ($\log_2$ haemagglutinin titre 9) treated (●) mice were assessed three days after a challenge with 4×10^8 SRBC. The primed mice received 1×10^6 SRBC thirty-seven days before challenge. The antibody-treated mice in addition received various amounts of IgG containing serum one day after the priming injection. Each point represents the arithmetic mean of the PFC response per spleen of six mice and the vertical lines represent one standard deviation of the reported mean.

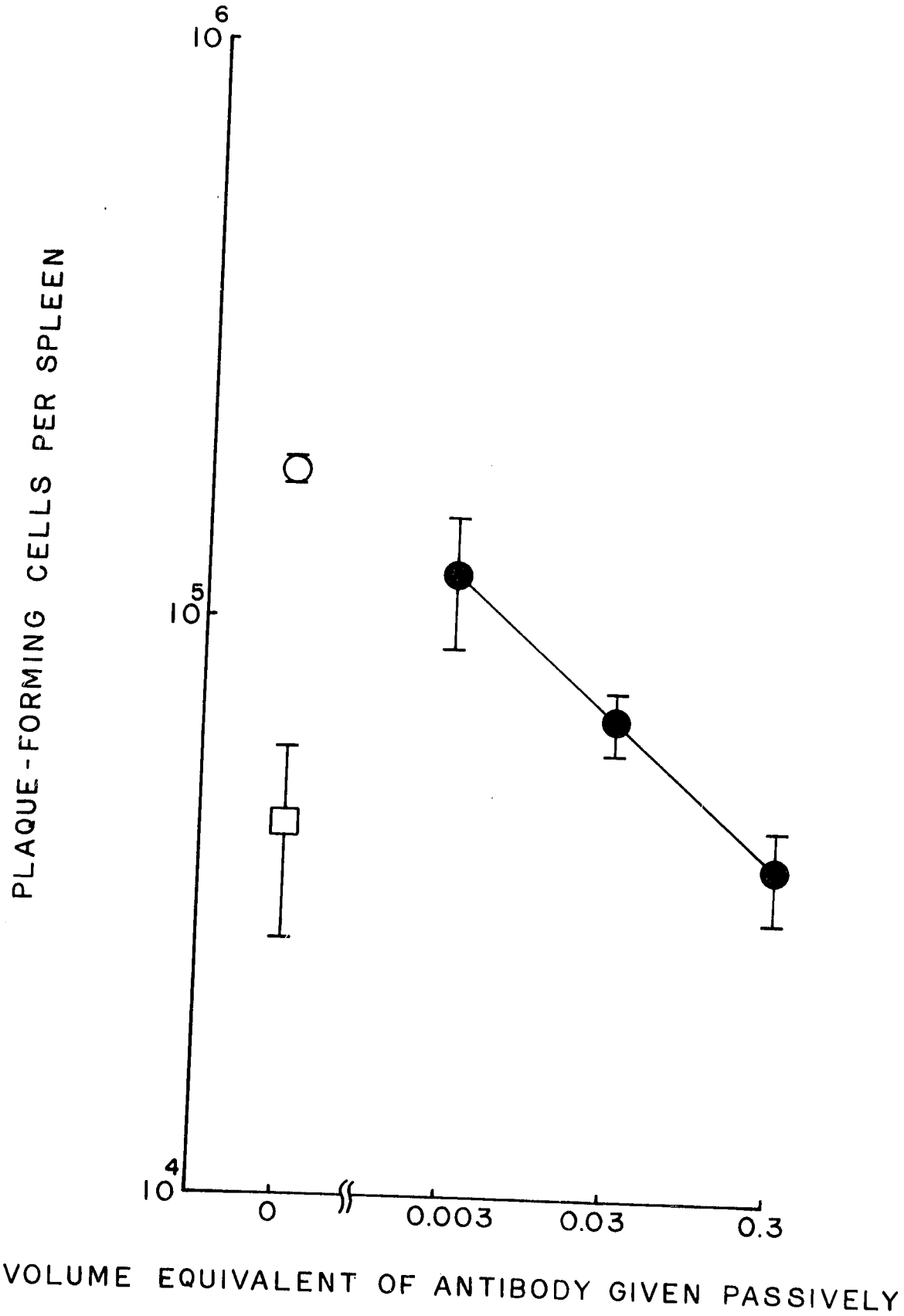
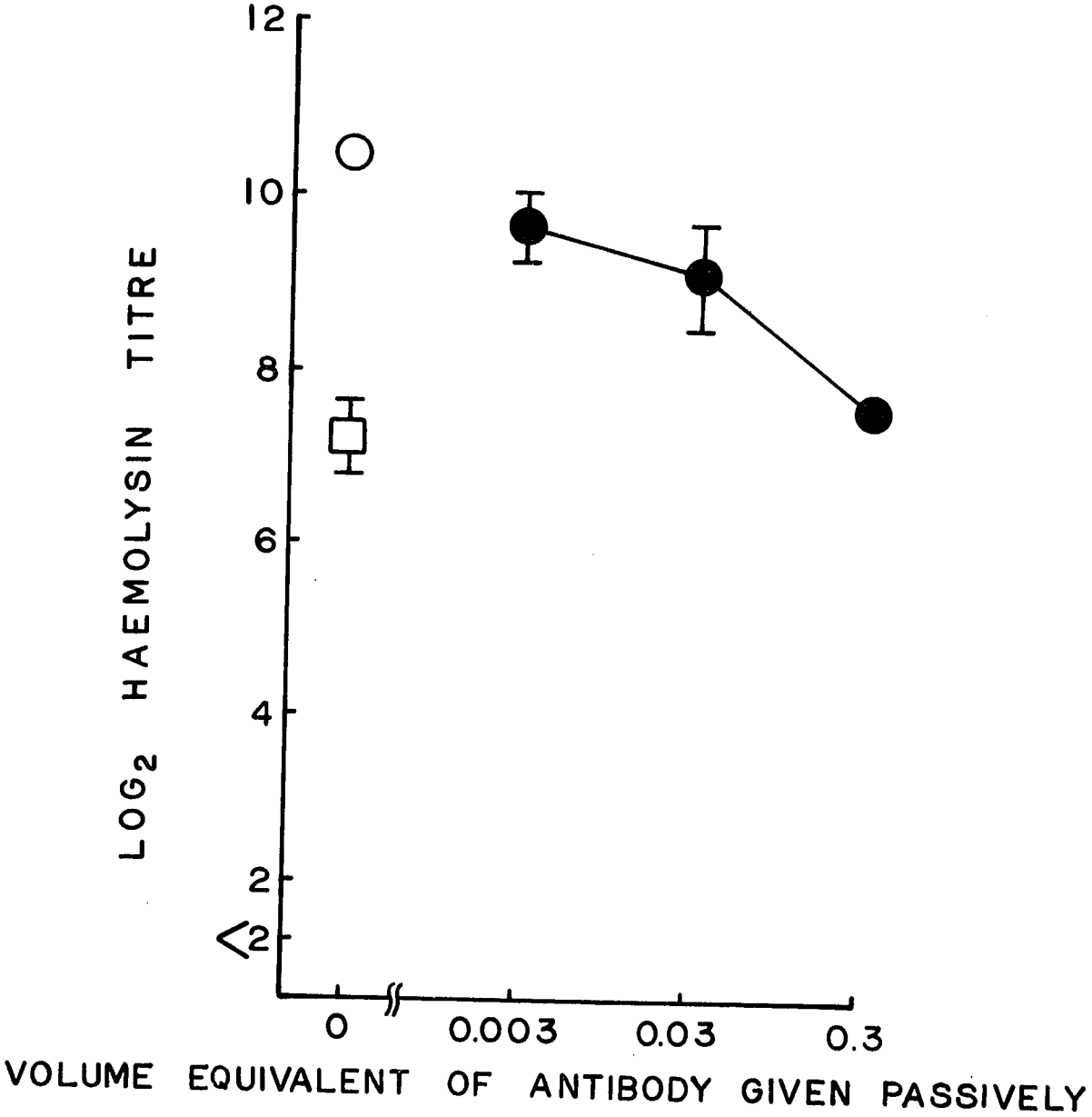

PLAQUE-FORMING CELLS PER SPLEEN
10^6
10^5
10^4
0
0.003
0.03
0.3
VOLUME EQUIVALENT OF ANTIBODY GIVEN PASSIVELY

FIGURE 6

SUPPRESSION OF SECONDARY HAEMOLYSIN RESPONSE BY IgG ANTIBODIES

The total haemolysin responses of normal (□), primed (○) and primed and antibody (log_2 haemagglutinen titre 9) treated (●) mice were measured three days after a challenge with 4×10^8 SRBC. The primed mice received 1×10^6 SRBC thirty-seven days before challenge. The antibody treated mice in addition received various amounts of IgG containing serum one day after the priming injection. Each point represents the arithmetic mean of the maemolysin titres (log_2) of six mice and the vertical lines represent one standard deviation of the reported mean.


12
10
8
6
4
2
<2
LOG2 HAEMOLYSIN TITRE
0
0.003
0.03
0.3
VOLUME EQUIVALENT OF ANTIBODY GIVEN PASSIVELY

tinguish between the inhibition of priming and the inhibition of secondary response.

4. SUPPRESSION OF IgM SECONDARY RESPONSE BY IgG ANTIBODIES GIVEN AFTER THE ESTABLISHMENT OF PRIMING. (CHALLENGE GIVEN AFTER TWENTY-THREE DAYS OF ANTIBODY ADMINISTRATION.)

Introduction

It is known that passive transfer of IgG antibodies soon after the antigen injection results in a suppressed primary response (Moller and Wigzell, 1965; Moller, 1969). IgG antibodies given shortly before antigen, or mixed with antigen before injection, result in a marked suppression of primary immune response (Rowley and Fitch, 1966; Uhr and Baumann, 1961). IgG antibodies have also been shown to suppress IgG secondary response.

In all these studies, however, a distinction between the suppression of memory and suppression of secondary response has not been made. In some earlier studies, a distinction between the suppression of IgM and IgG immune responses was not made. Since in the earlier studies, measurement of immune response was made in terms of serum antibodies, it was impossible to completely distinguish

between passively injected antibodies and endogenously produced antibodies. In the previous experiments (Figs. 5 and 6), it was not possible to distinguish between inhibition of IgM priming by IgG antibodies, and the direct effect of IgG antibody on IgM secondary response, without affecting priming. It was hypothesized that passively injected antibodies could affect the secondary response in one or more of the several possible ways:

a) It could inhibit priming only.

b) It could inhibit secondary response only.

c) It could inhibit priming as well as secondary response.

If we consider the first possibility, that is if IgG antibodies inhibit priming only, antibody injection given a considerable time after priming should not prevent the induction of a secondary response on challenge with antigen. On the other hand, if IgG antibodies inhibit IgM secondary response, then whatever time is allowed for priming, IgG antibodies given before challenge would inhibit secondary response.

Procedure

Mice were primed with a single intravenous injection of 5×10^5 SRBC. Varying amounts of IgG antibodies were given at Day ten, which is near the peak of priming. A

period of twenty-three days was allowed for the antibodies to decay. One group of primed mice did not receive antibody and served as a control group. A challenge injection of 4×10^8 SRBC was given to all groups of mice. A second control group was introduced at this stage, in which a group of normal mice received the challenge dose only and served as control primary response. Three days after the challenge, all mice were bled and sacrificed. The spleens obtained from the mice were assayed for PFC and the sera were titrated for total haemolysin activity.

Results

As can be seen in the PFC as well as in the total haemolysin response (Figs. 7 and 8), priming has been established. This is evident from the results as the primed control group gave a PFC response five times greater than that given by the non-primed control group. This difference is also reflected in the total haemolysin response. The primed control group gave a total haemolysin response which was about 4 $\log_2$ dilutions higher than the primary control group. It can also be seen that passive transfer of IgG antibodies resulted in a suppressed IgM secondary response. The suppression was markedly less in the total

FIGURE 7

EFFECT OF PASSIVE IgG ANTIBODIES GIVEN AFTER THE ESTABLISHMENT OF PRIMING ON SECONDARY IgM RESPONSE

The direct PFC responses of normal (□), primed (○) and primed antibody ($\log_2$ haemolysin titre 10) treated (●) mice were assessed three days after a challenge with 4×10^8 SRBC. The primed mice received 5×10^5 SRBC thirty-three days before challenge. The antibody treated mice, in addition, received various amounts of IgG containing serum ten days after priming injection. Each point represents the arithmetic mean of the PFC response per million spleen cells of five to seven mice and the vertical lines represent one standard deviation of the reported mean.

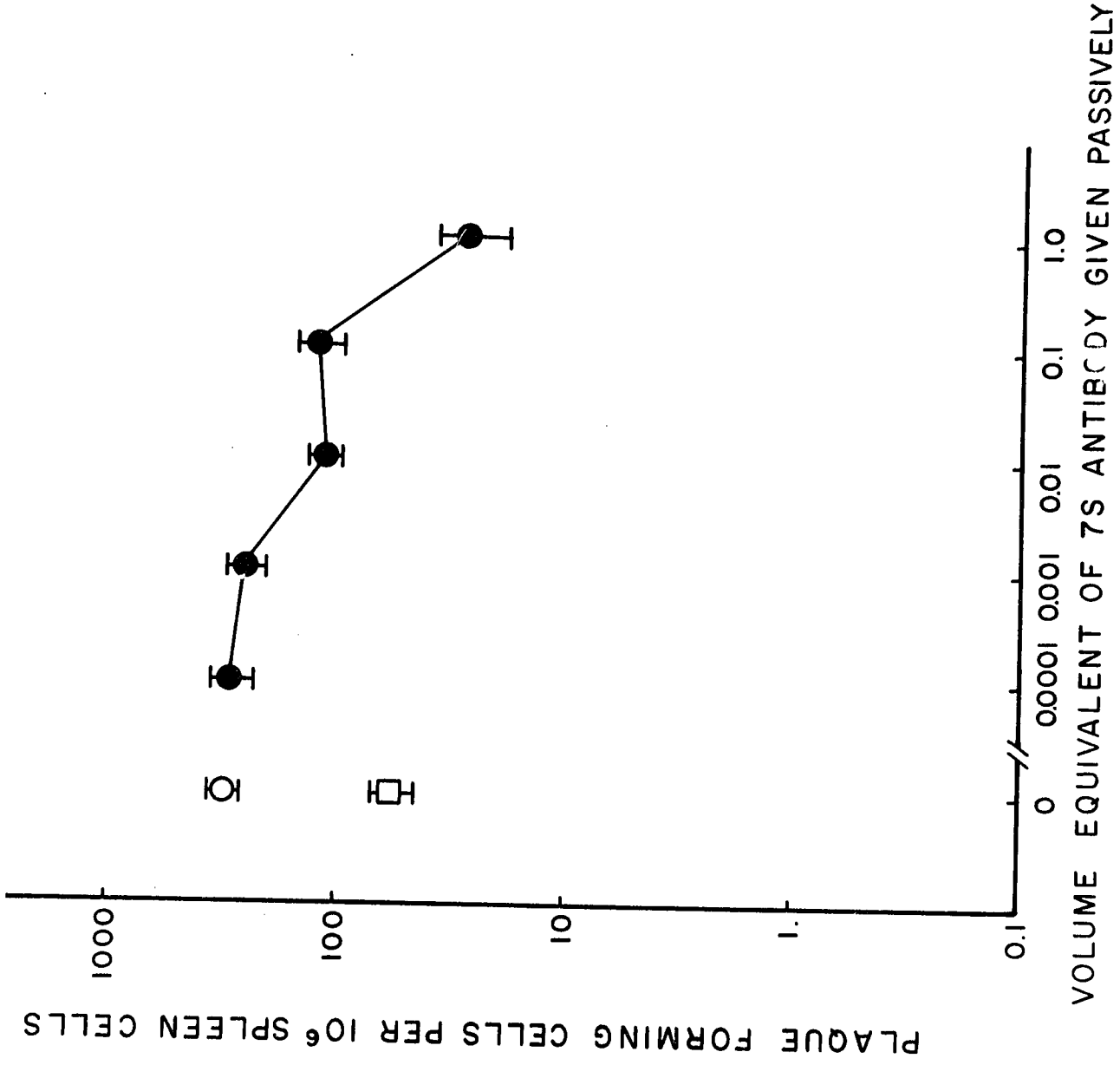
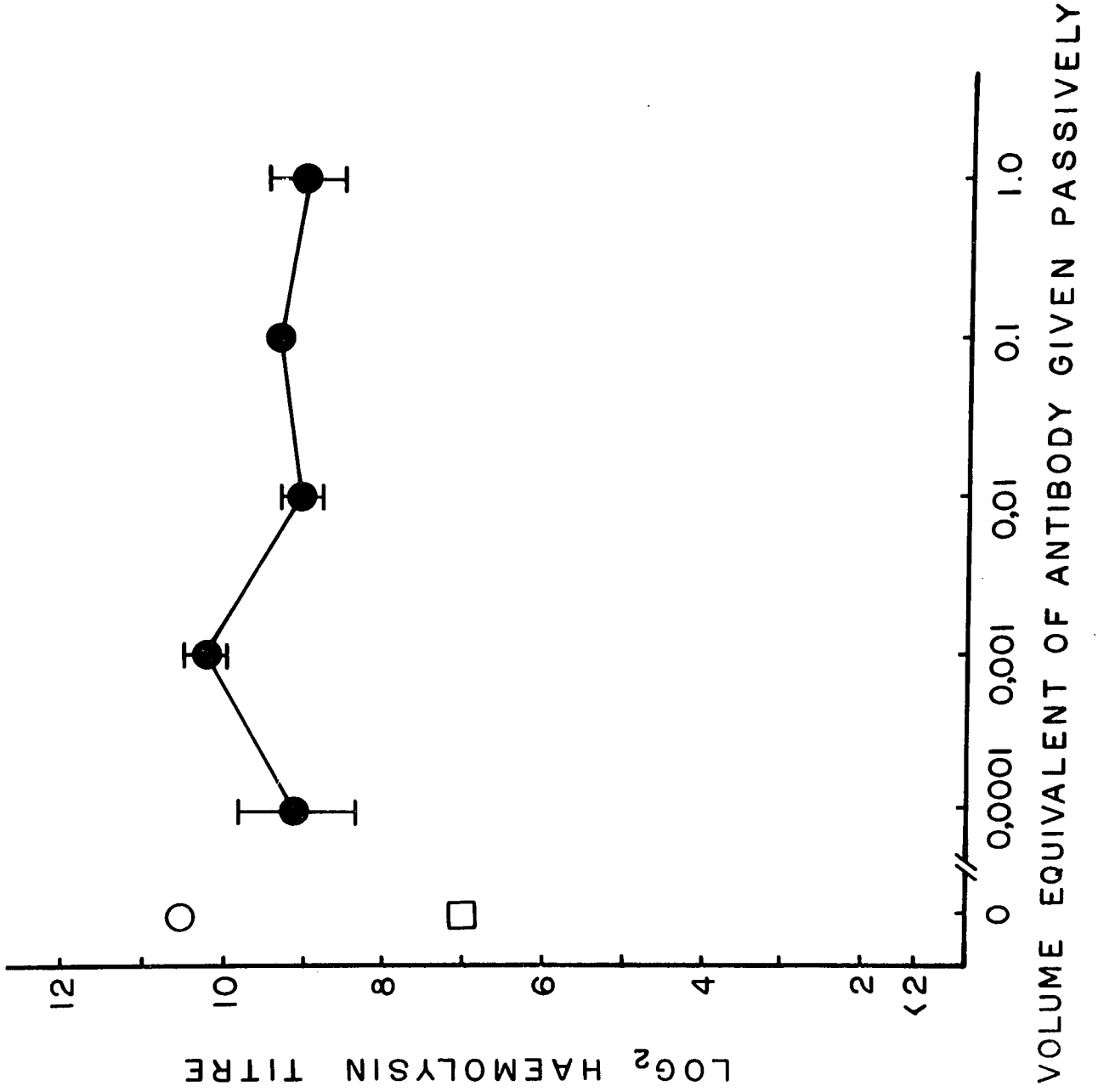

PLAQUE FORMING CELLS PER 10^6 SPLEEN CELLS
1000
100
10
1
0.1
0
0.0001
0.001
0.01
0.1
1.0
VOLUME EQUIVALENT OF 7S ANTIBODY GIVEN PASSIVELY

FIGURE 8

EFFECT OF PASSIVE IgG ANTIBODIES GIVEN AFTER THE ESTABLISHMENT OF PRIMING ON SECONDARY HAEMOLYSIN RESPONSE

The total haemolysin responses of normal (□), primed (○) and primed antibody (log_2 haemolysin titre 10) treated (●) mice were measured three days after a challenge with 4×10^8 SRBC. The primed mice received 5×10^5 SRBC thirty-three days before challenge. The antibody treated mice, in addition, received various amounts of IgG containing serum ten days after priming injection. Each point represents the arithmetic mean of the haemolysin titres (log_2) of five to seven mice and the vertical lines represent one standard deviation of the reported mean.


LOG2 HAEMOLYSIN TITRE
12
10
8
6
4
2
<2
0
0,0001
0,001
0,01
0.1
1.0
VOLUME EQUIVALENT OF ANTIBODY GIVEN PASSIVELY

haemolysin response compared to the PFC response. This may be due to an IgG secondary response which may be more resistant to the suppression by passive IgG transfer. Furthermore, it can be seen in Fig. 7 that the suppression of IgM secondary response, by the passive transfer of IgG antibodies, is greater when larger amount of antibodies is given. When 1 ml of antibodies was given passively, it resulted in a response which was slightly smaller than the primary response.

Discussion

Since passive transfer of antibodies was made at a time when priming was established, the effect of these antibodies could be on the IgM secondary response. It is unlikely that antibodies injected at this time could affect priming. Therefore, suppression in this case is most probably due to the effect of IgG on primed cells. However, the possibility of antigen-masking by antibody is not ruled out.

5. SUPPRESSION OF IgM PRIMARY AND SECONDARY RESPONSE BY IgG ANTIBODIES GIVEN AFTER THE ESTABLISHMENT OF PRIMING (CHALLENGE GIVEN SIMULTANEOUSLY WITH THE ANTIBODY.)

Introduction

The previous experiment (Fig. 7) indicated that, if IgG antibodies were present at the time of challenge, they could suppress IgM secondary response. It was thought that perhaps the sharp decline in IgM secondary response, soon after the peak of priming, had a similar mechanism behind it. In the previous experiment, the challenge was given thirty-three days after priming. Since a considerable decline in memory occurs during this period, it was desirable to study the effects of IgG antibodies when given at peak of priming (six days) simultaneously with the challenge. This could be done by priming mice with a low dose of antigen in order to minimize the antibodies produced as a result of priming antigen and their contribution towards suppression. The mice could then be challenged with either antigen alone, antigen mixed with antibodies or antigen and antibodies given separately but within a short time of each other.

Procedure

Mice were primed with a single injection of 5×10^5 SRBC. Six days later the mice were challenged with either 4×10^8 SRBC alone, or antigen mixed with varying amounts

of IgG antibodies. At the same time groups of normal mice were also challenged, with the same amount of antigen either alone, or antigen mixed with varying amounts of IgG antibodies. One group each of primed and non-primed mice was injected with the challenge antigen dose mixed with 0.1 ml of normal mouse serum, and served as control groups. A second control group of normal or primed mice received antigen alone. A group of normal mice and one group of mice which received the priming antigen only were included to serve as normal and primed background controls respectively. Three days after the challenge, all mice were bled and their spleens assayed for PFC response. The sera were titrated for total haemolysin activity.

Results

Fig. 9 shows the experimental results. The primary response was significantly suppressed when as small an amount of IgG antibody as 0.01 ml was mixed with antigen. Smaller amounts of IgG antibody did not significantly suppress the primary IgM response. One-tenth of an ml of normal mouse serum did not have any effect on the primary IgM response. Specific IgG antibodies suppressed the primary IgM response to the background level, when 0.1 ml was given

with challenge. The secondary IgM response was also significantly suppressed when 0.01 ml of IgG antibody was mixed with the challenge antigen. Larger amounts of IgG antibody mixed with challenge resulted in greater suppression, so that when 0.1 ml of IgG antibody was mixed with antigen, the secondary response was slightly above the primary response. When 0.1 ml of normal mouse serum was mixed with the challenge antigen, it did not have any effect on the secondary response. When IgG antibody was given separately, but within seconds of the challenge antigen, similar results were obtained, which can be seen in Figure 10. One-hundredth of one ml of IgG antibodies significantly suppressed the primary response. When 0.1 ml of specific IgG antibody was injected just before the challenge with antigen, primary response was suppressed about 50-fold. Similarly when 0.1 ml of specific IgG antibody was injected just before the challenge with antigen, the secondary response was suppressed about 10-fold. It can also be seen that 0.1 ml of normal mouse serum injected just before the challenges did not have any effect on the secondary response.

Discussion

It is evident from the results that very small amounts

FIGURE 9

SUPPRESSION OF PRIMARY AND SECONDARY IgM RESPONSE BY PASSIVE IgG ANTIBODIES MIXED WITH THE CHALLENGE

The direct PFC responses of normal (■) and primed (●) Swiss mice were assessed three days after the injection of challenge (4×10^8 SRBC) mixed with various amounts of IgG antibodies. The control responses of normal (□) and primed (○) mice three days after the challenge antigen alone were assessed at the same time. The control responses of normal (⬢) and primed (▲) mice receiving 0.1 ml of normal mouse serum with the challenge antigen and the background PFC of normal (⬡) and primed (▽) mice which did not receive challenge antigen were assessed at the same time as that of other groups. The primed mice were injected with 5×10^5 SRBC six days before challenge and the antibody or normal mouse serum was mixed with the challenge antigen before injection. Each point represents the arithmetic mean of PFC per million spleen cells of six mice and the vertical lines represent one standard deviation of the reported mean.

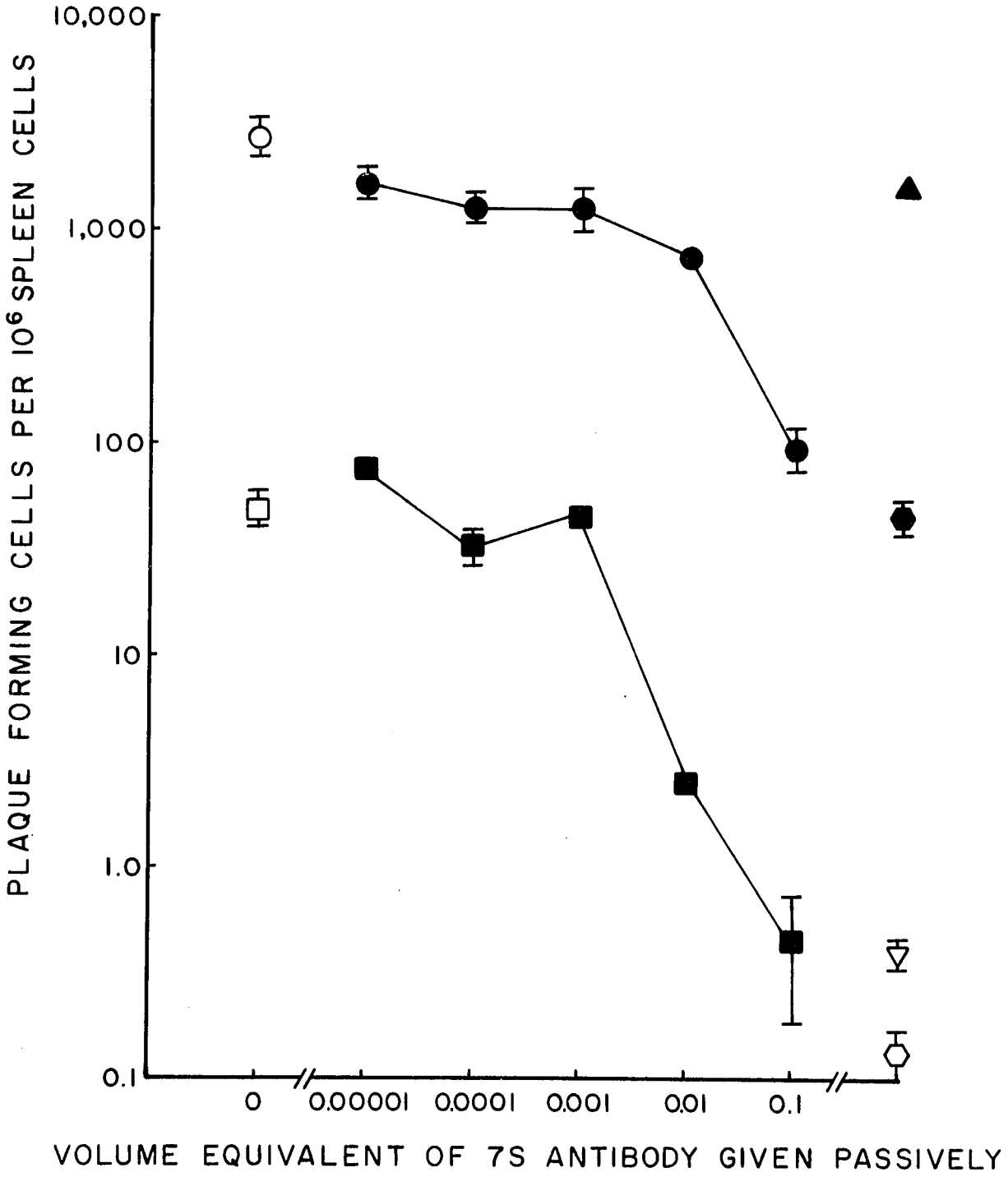
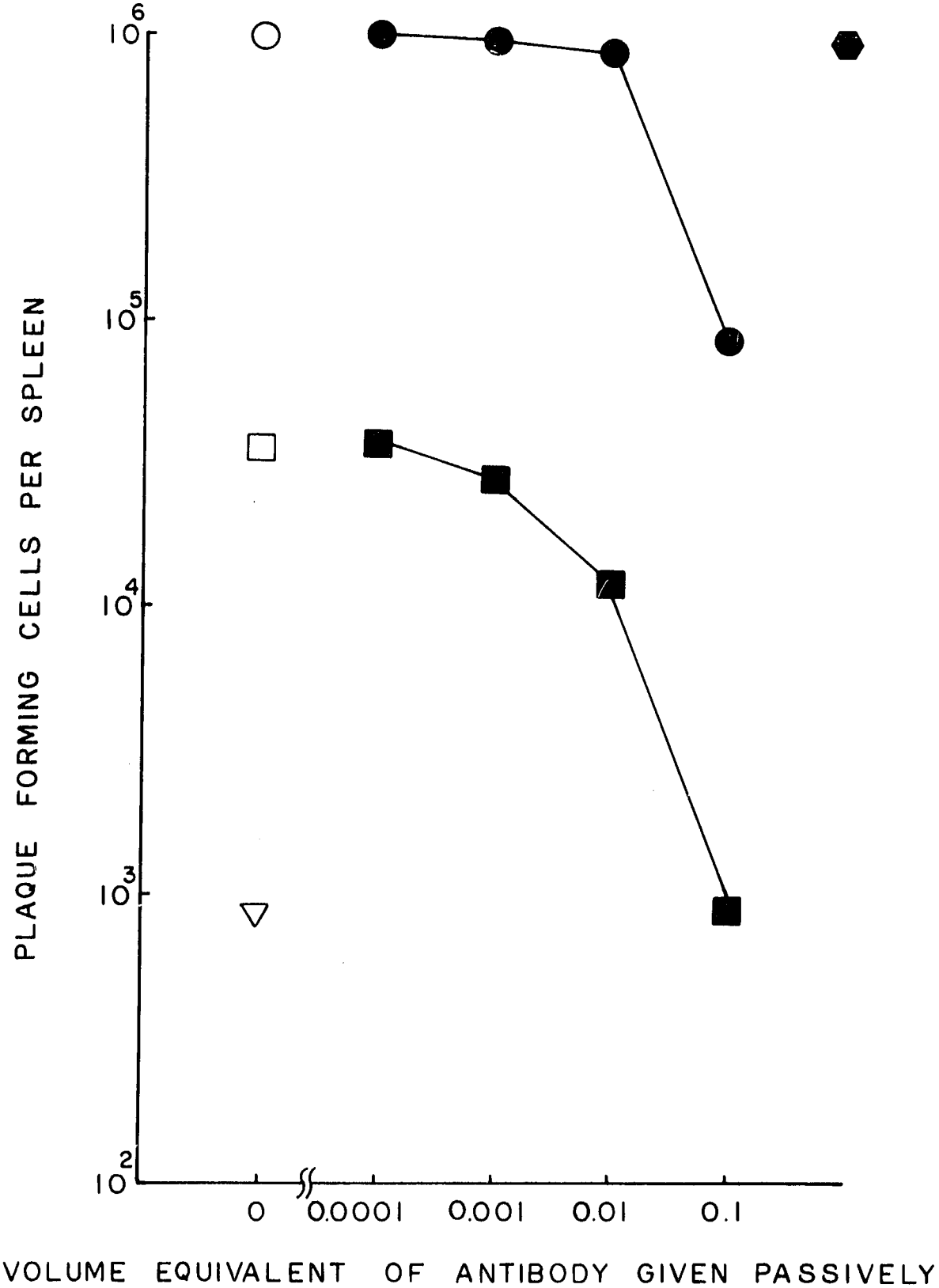

10,000
1,000
100
10
1.0
0.1
PLAQUE FORMING CELLS PER 10^6 SPLEEN CELLS
0
0.00001
0.0001
0.001
0.01
0.1
VOLUME EQUIVALENT OF 7S ANTIBODY GIVEN PASSIVELY

FIGURE 10

SUPPRESSION OF PRIMARY AND SECONDARY IgM RESPONSE BY IgG ANTIBODIES GIVEN SIMULTANEOUSLY WITH THE CHALLENGE

The direct PFC responses of normal (■) and primed (●) Swiss mice were assessed three days after injection of challenge (4×10^8 SRBC)and various amounts of IgG antibodies ($\log_2$ haemolysin titre 9). The control responses of normal (□) and primed (○) mice which received challenge but no antibody or primed mice which received challenge and 0.1 ml normal mouse serum (⬢) were assessed at the same time. The background PFC of primed mice which did not receive a challenge (▽) was also assessed with other groups. The primed mice were injected with 5×10^5 SRBC eight days before challenge and the antibody or normal serum was given separately but within seconds of the challenge. Each point represents the arithmetic mean of PFC per spleen of five to six mice.


PLAQUE FORMING CELLS PER SPLEEN
10^6
10^5
10^4
10^3
10^2
0
0.0001
0.001
0.01
0.1
VOLUME EQUIVALENT OF ANTIBODY GIVEN PASSIVELY

of IgG antibody can inhibit both the primary response as well as the expression of priming. Moreover, when the degree of IgG antibody-induced suppression of IgM secondary response is compared with the degree of suppression of primary IgM response, it is seen that secondary response is relatively less suppressed than the primary response. This resistance to suppression may be due to a larger number of immunocompetent cells in the primed animals compared to non-primed animals. It is also possible that this difference is due to qualitative differences between virgin immunocompetent cells and memory cells. For instance, it is possible that primed cells or memory cells have increased affinity for antigen compared to the non-primed cells and therefore are more easily stimulated by antigen. On the other hand, it is also possible that the primed cells are more difficult to suppress by IgG antibody. The increased suppression of secondary response observed in this experiment compared to the previous experiment (Fig. 7) might be a quantitative one, in that more antibody is available at the time of challenge in this experiment because of the lack of decay.

6. ANTIGEN DOSE AND PRIMARY IgM AND IgG PFC RESPONSE

Introduction

IgM memory has been studied by a number of workers and conflicting results have been reported. Since different antigen doses were used for priming by various workers, it is possible that the differences in results relate to the priming antigen doses. It was therefore important to study the effect of various priming doses of antigen on IgM secondary response. In order to carry out this study, it was considered desirable to study the primary response to varying antigen doses before these doses were used for priming.

Procedure

Mice were given a single intravenous injection of varying amounts of antigen. Eight days after the antigen injection, mice were killed by cervical dislocation. A group of normal mice was used for background responses. The spleens of the mice were assayed for direct (IgM) and indirect (IgG) PFC response. For developing the indirect plaques, rabbit anti-mouse globulin serum was used.

Results

Figures 11 and 12 show the results of two such experiments. As can be seen, the primary IgM and IgG response

FIGURE 11

THE ANTIGEN DOSE FOR MAXIMUM PRIMARY IgM AND IgG PFC RESPONSE

The IgM (□) and IgG (⬡) PFC responses of Swiss mice were assessed eight days after a single injection of various doses of antigen. Each point represents the arithmetic mean of the PFC per spleen of three mice.

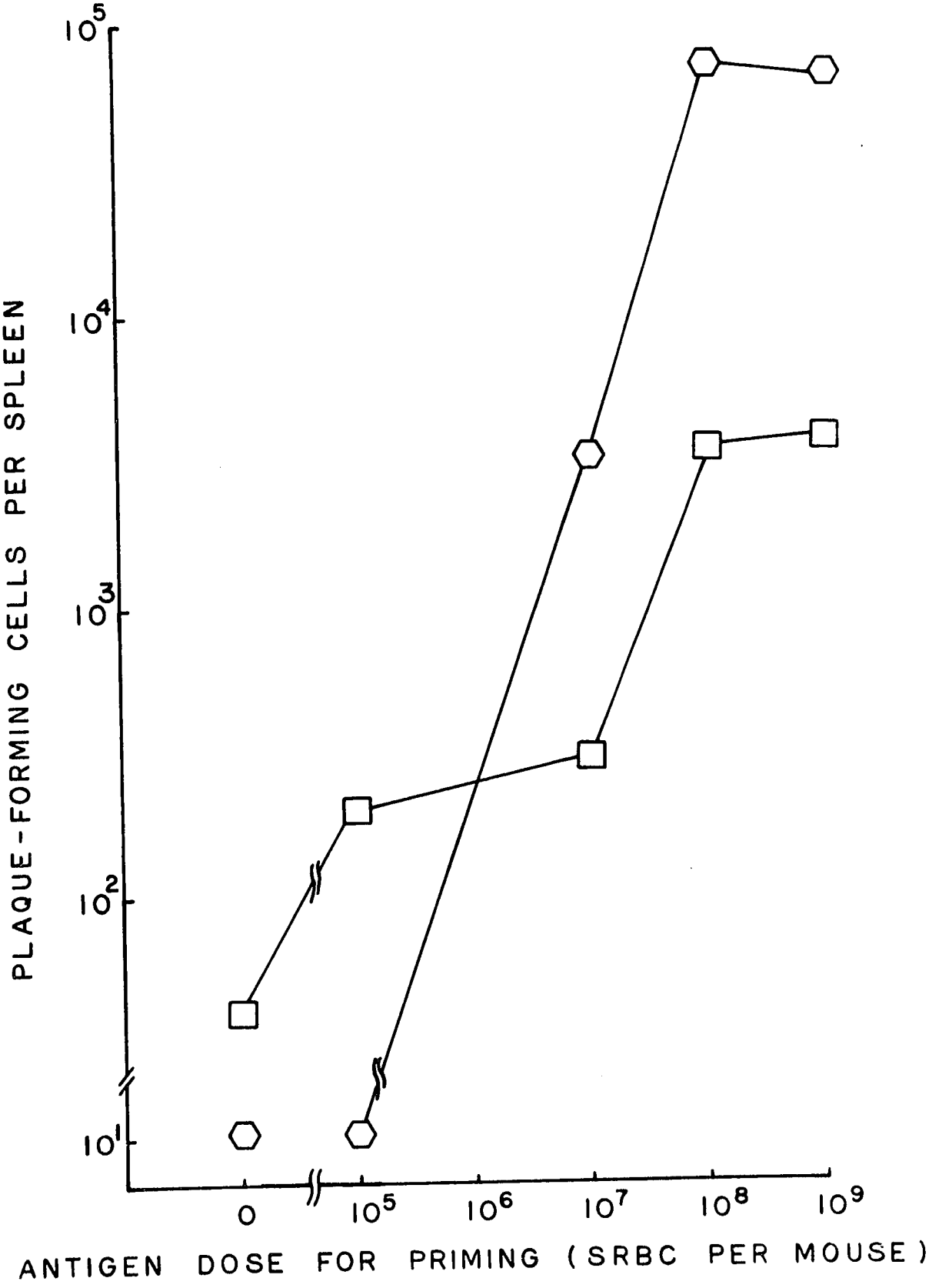
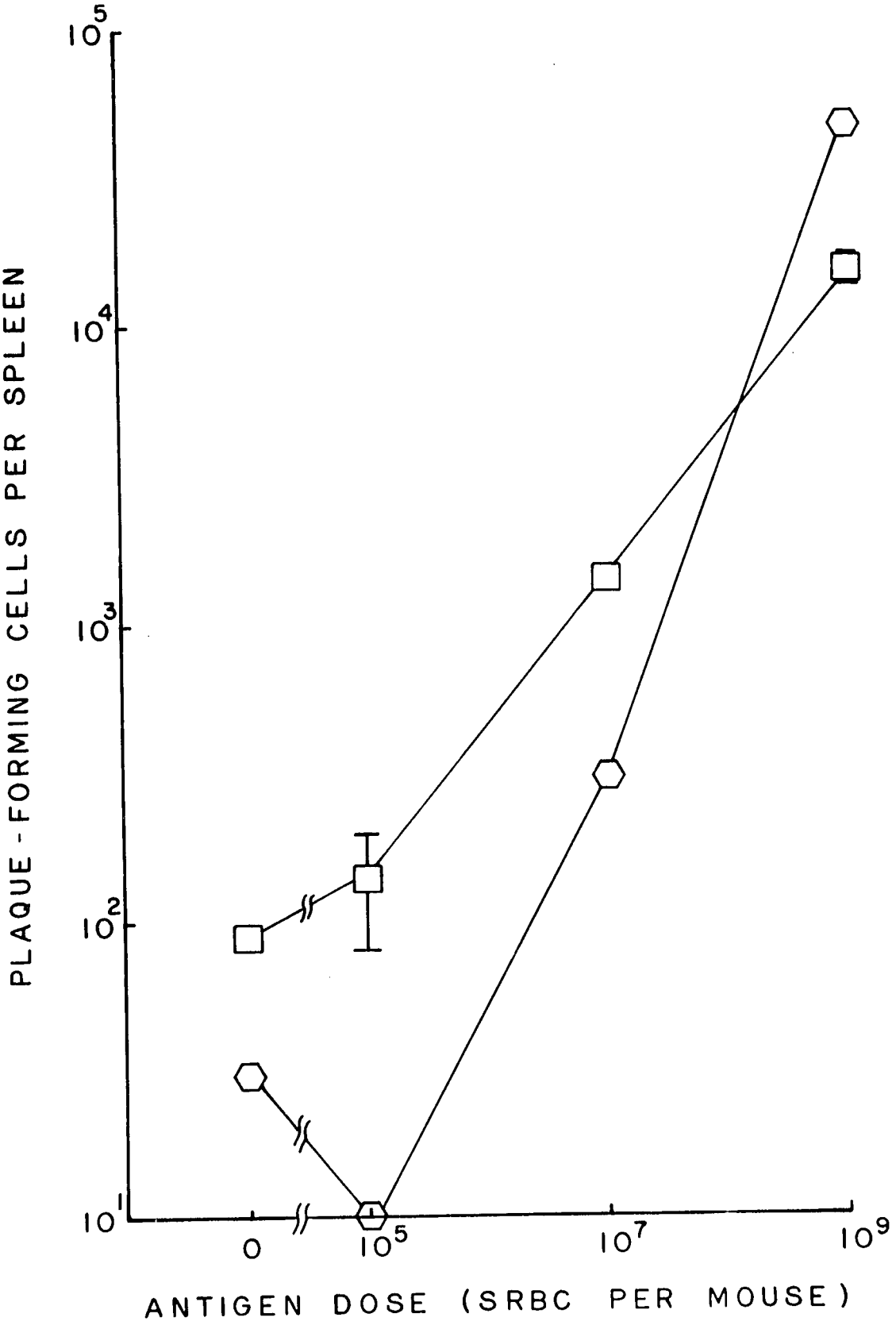

PLAQUE-FORMING CELLS PER SPLEEN
10^5
10^4
10^3
10^2
10^1
0
10^5
10^6
10^7
10^8
10^9
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

FIGURE 12

A DOSE-RESPONSE STUDY OF PRIMARY IgM AND IgG PFC RESPONSE

The IgM (□) and IgG (⬡) PFC responses of Swiss mice were assessed eight days after a single injection of various doses of antigen. Each point represents the arithmetic mean of PFC per spleen of three to five mice. The vertical lines represent one standard deviation of the reported mean.


PLAQUE-FORMING CELLS PER SPLEEN
10^5
10^4
10^3
10^2
10^1
0
10^5
10^7
10^9
ANTIGEN DOSE (SRBC PER MOUSE)

was significantly above the normal background response when an injection of 10^7 SRBC was given. With a further ten-fold increase in the antigen dose, there was a further increase in the response. When the antigen dose was increased to 10^8 SRBC, both the IgM and IgG response reached maximal levels in one experiment (Fig. 11). It can be seen, moreover, that there was a marked increase in IgG response when the antigen dose was increased from 10^7 to 10^9 SRBC.

7. EFFECT OF PRIMARY ANTIGEN DOSE ON SECONDARY IgM RESPONSE

Introduction

It has been mentioned earlier that the decline in memory may be due to the induction of IgG antibodies. As has been seen in the last experiment (Figs. 11 and 12), a high antigen dose induced a high IgG response and a low antigen dose gave a low IgG response. If IgG is responsible for a decline in IgM secondary response, then one would expect an inverse relationship between the dose of antigen used for priming and the IgM secondary response obtained beyond the point where priming antigen induces sufficient amounts of IgG antibodies.

Procedure

Mice were primed with varying antigen doses. Eight days later all groups of mice were challenged with a constant optimal antigen dose. A group of normal mice was also injected with the challenge dose and served as a control primary response. Three days after the challenge, all mice were bled and sacrificed. The spleens of these mice were assayed for PFC response, while their sera were titrated for total haemolysin activity.

Results

It can be seen that priming with 10^5 SRBC significantly increased the secondary IgM response (Fig. 13). The secondary response continued to increase, with increasing priming dose. The largest secondary response was obtained when 10^7 SRBC was used for priming. When an antigen dose larger than 10^7 SRBC was used for priming, a decrease in secondary immune response was obtained. When 10^9 SRBC were used for priming, the secondary response obtained was less than one-tenth of that obtained by the optimum priming dose. Similar results were obtained when the sera from these mice were titrated for total haemolysin activity (Fig. 14).

FIGURE 13

THE EFFECT OF PRIMING ANTIGEN DOSE ON IgM SECONDARY RESPONSE

The direct PFC responses of Swiss mice primed with various doses of antigen (○) were assessed three days after a challenge with 4×10^8 SRBC. The control response of normal mice three days after the challenge is indicated by the open square (□). The priming with various doses was carried out eight days before the challenge. Each point represents the arithmetic mean of PFC per spleen of five mice. The vertical lines represent one standard deviation of the reported mean.

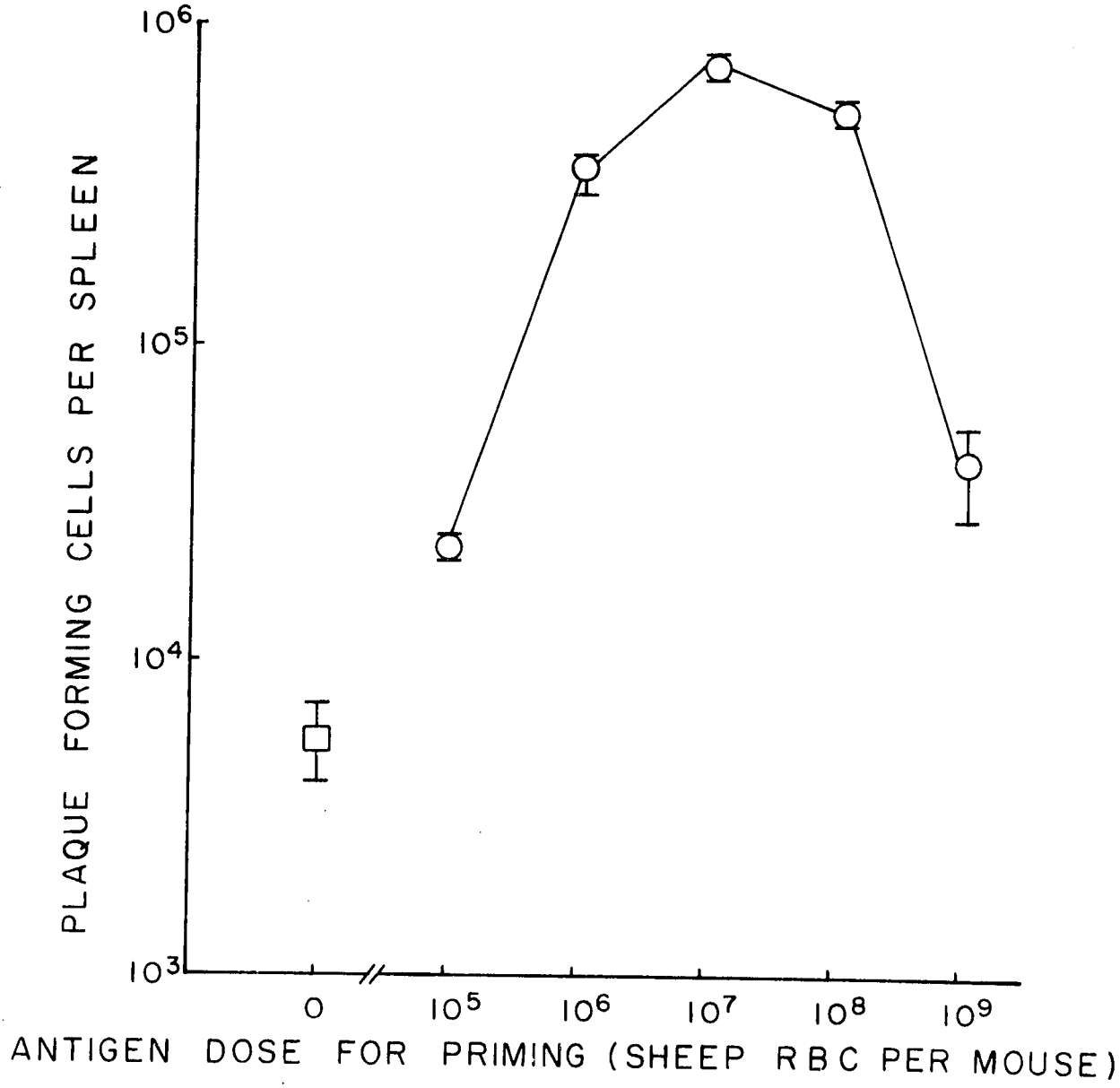
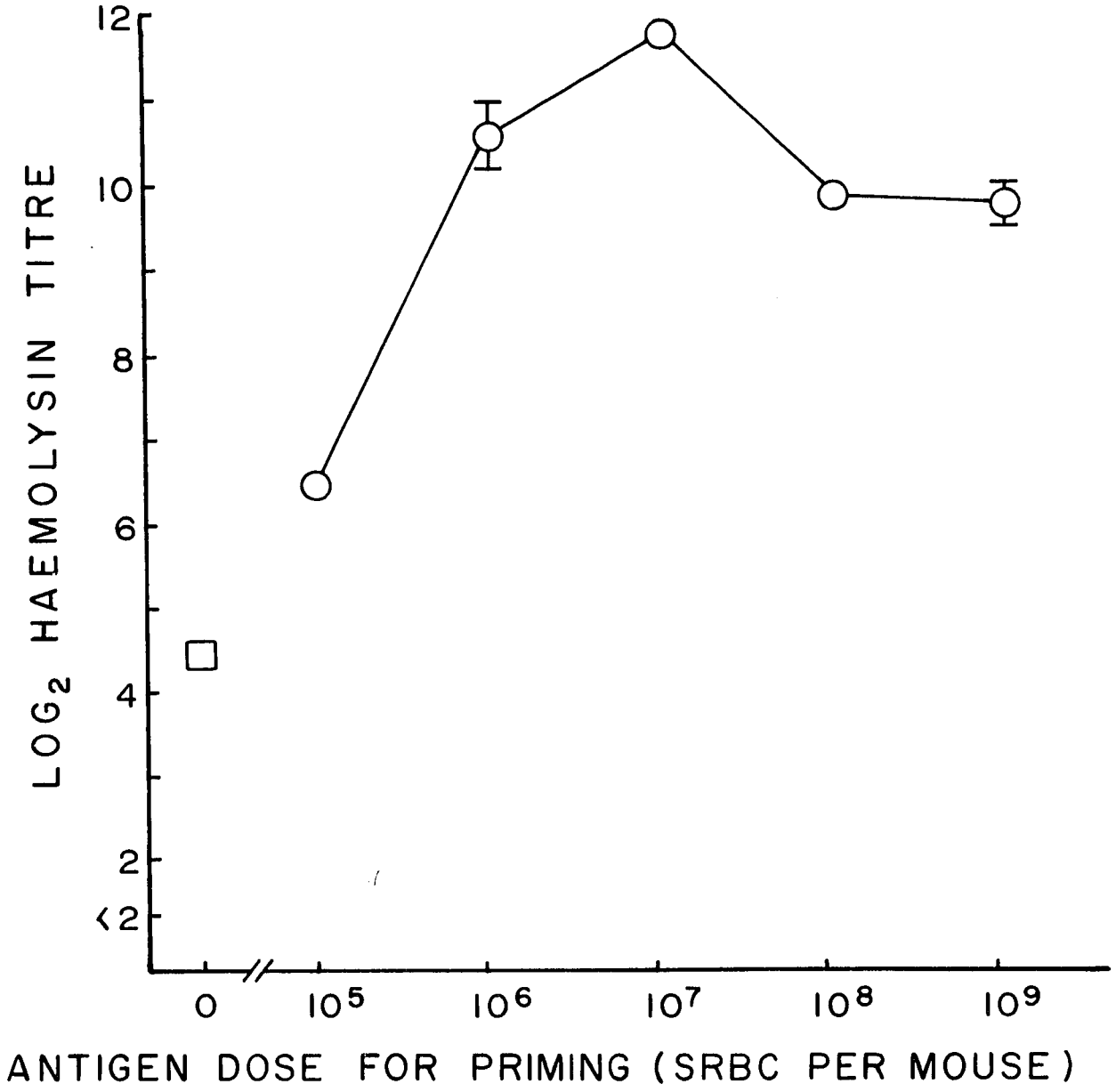

10^6
10^5
10^4
10^3
PLAQUE FORMING CELLS PER SPLEEN
0
10^5
10^6
10^7
10^8
10^9
ANTIGEN DOSE FOR PRIMING (SHEEP RBC PER MOUSE)

FIGURE 14

THE EFFECT OF PRIMING ANTIGEN DOSE ON THE SECONDARY HAEMOLYSIN RESPONSE

The total haemolysin responses of mice primed with various doses of antigen (○) and of normal mice (□) were assessed three days after a challenge with 4×10^8 SRBC. The primed mice received the priming dose eight days before the challenge. Each point represents the arithmetic mean of haemolysin titres ($\log_2$) of five mice and the vertical lines represent one standard deviation of the reported mean.


12
10
8
6
4
2
<2
LOG2 HAEMOLYSIN TITRE
0
10^5
10^6
10^7
10^8
10^9
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

Discussion

With increasing amounts of antigen for priming, a number of possibilities exist. If an animal has a limited pool of virgin immunocompetent cells, and these cells give rise to memory cells on antigenic stimulation, it can be expected that, with increasing doses of antigen, a point would be reached where a maximal number of the virgin cells are transformed into memory cells upon antigenic stimulation. Beyond this point, a further increase in the antigen dose would not increase the amount of memory. According to X-Y-Z scheme of Sercarz and Coons (1962), however, the virgin X-cell can undergo two types of differentiation under the influence of antigen. Firstly, it can differentiate to Y-cell or memory cell and then to Z-cell or antibody-forming cell, or, secondly, it can differentiate to Z-cell directly. When a large antigen dose is used for priming, there would be a small pool of Y-cells and a large pool of Z-cells. According to the first possibility, there would be an initial increase in the Y-cell compartment with increasing antigen dose, and beyond the peak, the Z-cell compartment will increase with the concomitant decrease in the Y-cell compartment. The situation is, however, further

complicated by the fact thatincreasing antigen dose results in an increased IgG response which may inhibit secondary response. The decline in secondary response, when a large dose of antigen is used for priming, therefore can be the result of any one or more of the three possibilities: a) differentiation of X-cells directly to Z-cells; b) expenditure of Y-cell compartment under the influence of excess antigen; c) suppression of secondary response by IgG antibodies produced.

B. EXPERIMENTS IN γ-IRRADIATED ANIMALS

1. KINETICS OF PRIMARY AND SECONDARY IgM RESPONSE IN IRRADIATED HOSTS

Introduction

In previous experiments, it was shown that: - 1. The peak priming occurs approximately one week after the priming antigen injection. 2. IgG antibodies suppress secondary IgM response when given either before or after the establishment of peak priming. 3. The optimum dose for peak priming of IgM response is 10^7 SRBC. High doses of antigen for priming lead to a suppression of secondary IgM response. However, the following questions were still unanswered: a) Was the decline in memory after Day eight (Fig. 2) due to IgG production or absence of memory cells? b) Did passive antibody inhibit priming? c) Did the large dose of antigen for priming result in a small number of memory cells or were memory cells present but were functionally blocked? In order to shed some light on the above questions, the following series of cell transfer experiments were performed, using γ-irradiated recipients. In the previous section, IgM secondary response was studied

in the presence of antibodies produced due to the priming antigen dose. In the present experiments this was avoided by transferring washed cells from primed animals along with challenge antigen to irradiated recipients. Lethally irradiated mice are incapable of giving an immune response on antigenic challenge. These mice would die within two weeks if not protected by spleen or bone marrow cells. The protection afforded by spleen or bone marrow injection into these animals is due to the haematopoietic stem cells in these cell preparations. Spleen cells from immunized or non- immunized mice, transferred to lethally irradiated hosts, do not give an immune response, unless they are challenged with antigen. As has already been mentioned, many of the questions asked in the previous section could not be answered in the intact animals. In order to use cell transfer system to answer these questions, it was necessary to study the kinetics of the response in this system. Experiments were carried out to establish the time course of immune response given by primed or non-primed cells in lethally irradiated hosts.

Procedure

Mice were primed with a single intravenous injection

of 5×10^5 SRBC. Ten days after priming, one group of primed and a group of normal mice were killed by cervical dislocation. Cell suspensions were prepared from their spleens. The cell preparations were washed a number of times in ice cold Medium 199. Lethally irradiated (800 rads) hosts received either 1×10^7 primed or non-primed cells, along with 4×10^8 SRBC. The recipient mice were killed at various time intervals between four to seven days after the cell transfer, and their spleens assayed for PFC response.

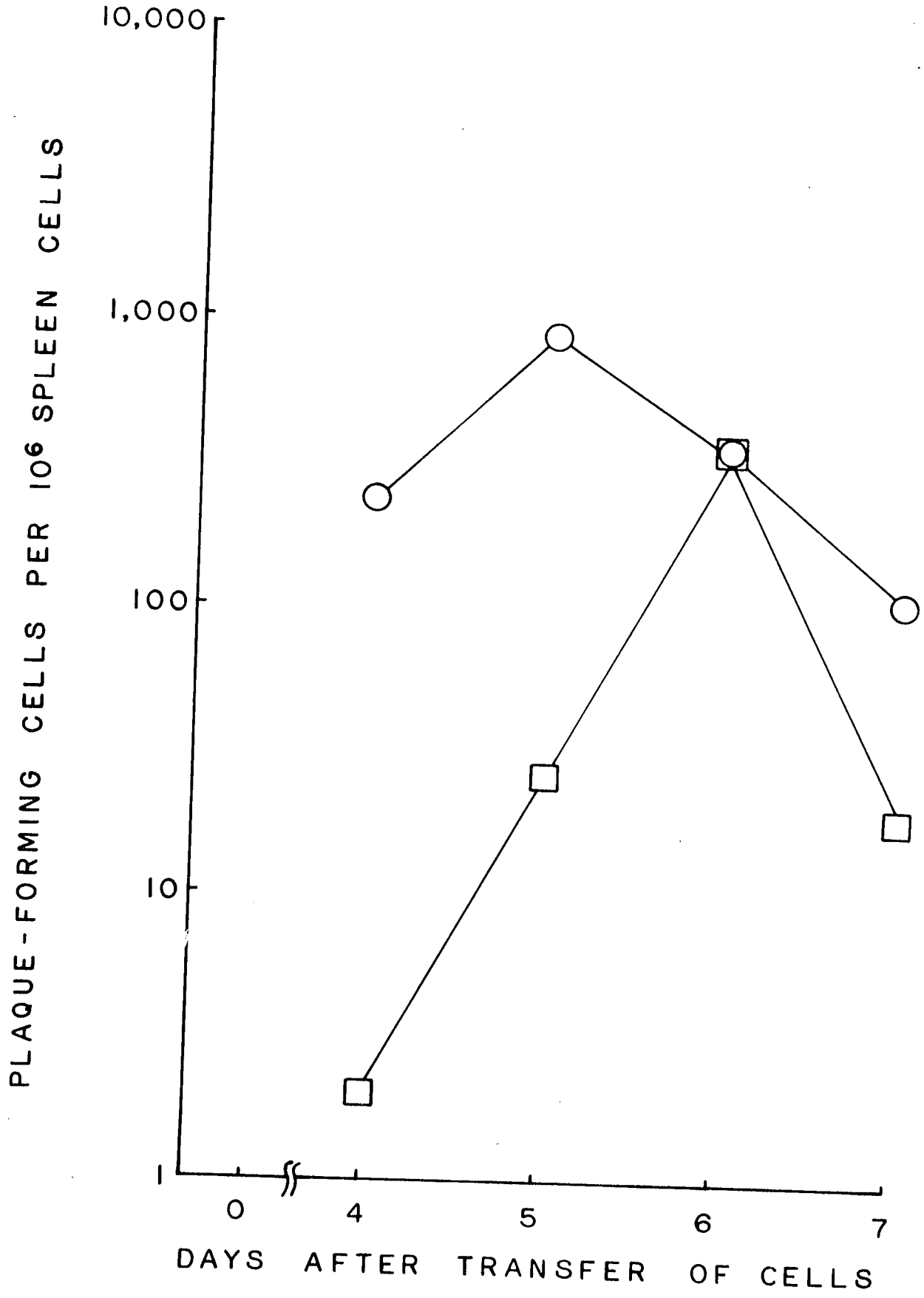
Results

In Fig. 15, it can be seen that, in general, primed cells showed a higher immune response than non-primed cells. Also primed cells showed their peak response a day earlier than non-primed cells. Thus on Day six when non-primed cells were showing a peak response, the response of primed cells had already declined. Therefore in all subsequent cell transfer experiments where the immune response of primed and non-primed cells were being compared, assays were performed at Day five. Non-primed cells gave a very small immune response four days after the transfer into irradiated hosts. Thereafter the response increased rapidly

FIGURE 15

KINETICS OF PRIMARY AND SECONDARY IgM RESPONSE UPON CELL TRANSFER INTO IRRADIATED HOSTS

The direct PFC response of lethally irradiated mice was assessed at various times after transfer of 10^7 normal (□) and primed (○) spleen cells along with 4×10^8 SRBC. The primed cells were obtained from the spleens of donor mice which received 5×10^5 SRBC eleven days before sacrifice. Each point represents the arithmetic mean of PFC per million spleen cells of four recipients.


10,000
1,000
100
10
1
PLAQUE-FORMING CELLS PER 10^6 SPLEEN CELLS
0
4
5
6
7
DAYS AFTER TRANSFER OF CELLS

and reached a peak on Day six. Beyond the peak, there was a sharp decline in the PFC response and at Day seven it was already less than 1/10th of the immune response on Day six. Primed cells gave a response on Day four which was one hundred times larger than that given by non-primed cells. There was a further five-fold increase in the response of the primed cells during the next twenty four hours. On Day five the primed cells reached the peak of response, and beyond this their response declined rapidly. On Day six, the response given by the primed cells was about the same as on Day four. At this time there was no difference between the response given by primed or non-primed cells.

2. A TIME COURSE OF IgM PRIMING STUDIED IN IRRADIATED HOSTS

Introduction

It has been stated that the decline in IgM memory in the intact animal may be due to the production of IgG antibodies. These antibodies then could inhibit the secondary IgM response. It has been shown (Cunningham, 1969) that transfer of cells from mice primed for as long as five months results in a good IgM secondary response. It was

desirable to confirm these results in order to carry on further experiments on IgM memory. It is possible that in intact animals primed for a long time, memory cells are present but inhibited from responding to antigenic challenge. This inhibition may be caused by IgG antibodies produced as a result of priming antigen. If this is the case, then cells from animals primed for a long time would be expected to give a good secondary response on transfer and antigenic challenge in irradiated recipients.

Procedure

Mice were primed with a single intravenous injection of 5×10^5 SRBC. At different times after this injection, mice were killed and cell suspensions prepared from their spleens. The cells were washed three times in large volumes of Medium 199 and resuspended in the same medium. Groups of irradiated mice received 2×10^7 primed cells per mouse along with 4×10^8 SRBC. One group of mice which received 2×10^7 cells from non-primed animals along with 4×10^8 SRBC served as the control. Five days later, the recipient mice were killed and assayed for their immune response.

Results

Fig. 16 shows the experimental results. It can be seen that a significant priming was established at Day two. The degree of priming increased with time and reached a plateau at Day six. After this the response did not vary a great deal up to Day eighty, except at Day eight where it was depressed. This might be due to experimental variations. Unlike the secondary responsiveness of the intact animals (Fig. 2), there was no decline in the responsiveness of cells obtained from animals primed for a long period of time.

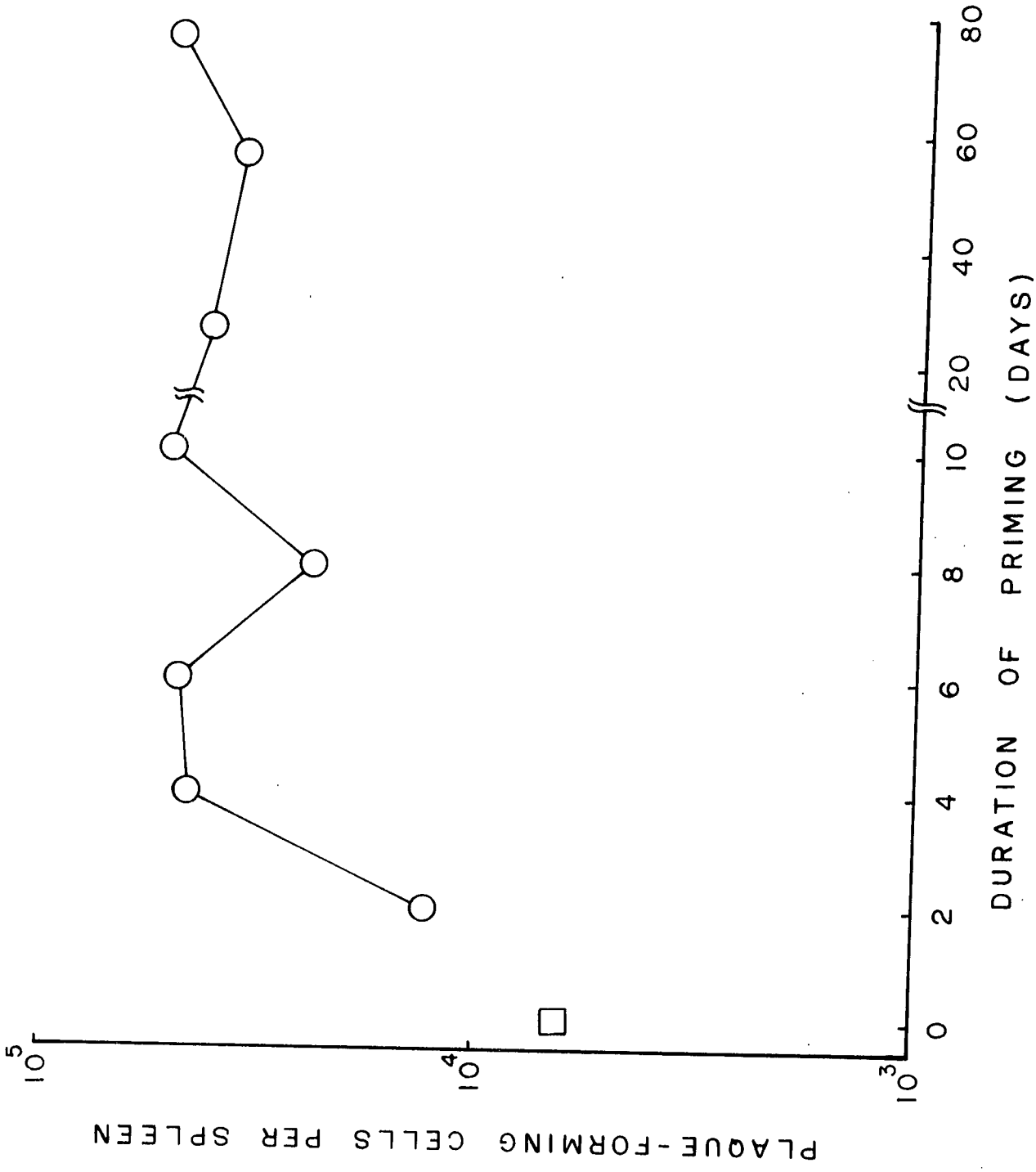
Discussion

The lack of decline in the response upon transfer of cells indicates that the memory cells are present in a primed animal a long time after the first injection, but for some reason do not respond in the intact animal. It clearly shows that the lack of secondary response in the intact animal is not due to the absence of memory cells, but due to their functional inactivity. It seems very likely that the memory cells in the intact animal are inhibited from responding upon antigenic challenge by some

FIGURE 16

THE DEVELOPMENT OF IgM MEMORY STUDIED BY CELL TRANSFER

The direct PFC response of lethally irradiated mice was assessed five days after transfer of 2×10^7 cells from normal donors (□) and donors primed with 5×10^5 SRBC for various times (○). The challenge dose of 4×10^8 SRBC was given to all groups of mice simultaneously with the cell transfer. Each point represents the arithmetic mean of PFC per spleen of six recipient mice.


PLAQUE-FORMING CELLS PER SPLEEN
10^5
10^4
10^3
0
2
4
6
8
10
20
40
60
80
DURATION OF PRIMING (DAYS)

factor(s) in the animal a few weeks after the priming. There may be several possible factors which could give such a result. It is possible that soon after priming, memory cells migrate to anatomical sites, where antigen can not easily stimulate them. It may well be that some factor or factors such as IgG antibodies or antigen-antibody complexes produced as a result of antigenic stimulation, block the memory cells from responding to a secondary challenge.

The responsiveness of the cells upon transfer could be attributed to their homing to sites where antigen can stimulate them, or due to the washing away of the blocking factors. The next two experiments were therefore performed in an attempt to delineate the role of IgG in suppression of priming and of primed cells.

3. EFFECT OF PASSIVE TRANSFER OF IgG ANTIBODIES TO THE CELL DONORS ON IgM PRIMING

Introduction

In the last experiment, cells transferred from donors which were given a priming injection several weeks before gave a good secondary response (see Fig. 16). This responsiveness of cells did not decline if the duration of priming was increased. This finding was unlike the results

when challenge was given to the intact animals (Fig. 2), there being a sharp decline in the response with increasing interval of time. These results and the suppressive effects of IgG antibodies on IgM response (Figs. 7, 9 and 10) suggested that, in intact animals, expression of IgM memory may have been blocked by IgG antibodies produced as a result of priming antigen. However, from the experiments in the intact animals, it could not be said whether the suppressed response was due to the inhibition of priming or to its expression. It was therefore postulated that if IgG antibodies inhibit IgM priming in the intact animals, it should be possible to achieve this inhibition by passive transfer of IgG antibodies soon after priming antigen dose. Transfer of cells from such treated hosts should then result in a poor secondary response. If, however, IgG antibodies inhibit the expression of priming, then cells from such animals should give a good secondary response.

Procedure

Mice were given an intravenous injection of 1×10^6 SRBC for priming. One day after the antigen injection, these mice were given various amounts of IgG antibody or its $F(ab')_2$ fragment. The control group did not receive

antibody or $F(ab')_2$. Thirty-six days later, the mice were sacrificed and 2×10^7 of their spleen cells transferred into irradiated recipients along with 4×10^8 SRBC. (The spleen cells were washed three times in a large volume of Medium 199 and made up to a final suspension to give 2×10^7 cells in 0.2 ml volume.) At the same time, equal number of cells from normal donors, prepared in identical way, were also transferred into a group of recipients. This group served as control primary response.

Results

It can be seen in Fig. 17 that cells from mice, primed for an interval of thirty-seven days, gave a good secondary response as compared to the response of cells from non-primed animals. The response was more than ten-fold higher. No significant suppression of priming was seen in groups receiving cells from mice which were given IgG antibodies or $F(ab')_2$ antibodies in addition to the priming antigen. Whether the donor mice were treated with intact IgG or its $F(ab')_2$ fragment, the response of the recipients was the same.

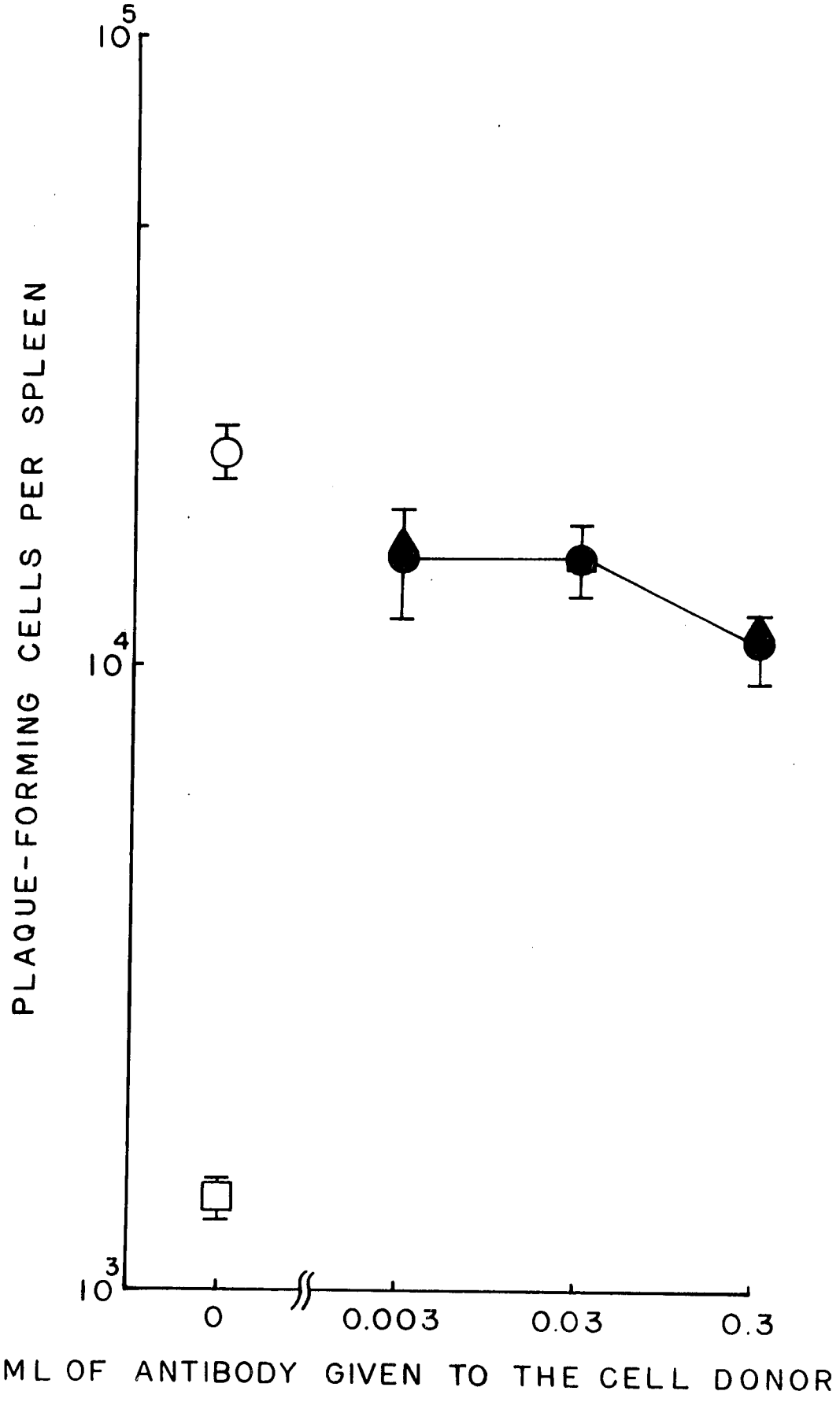
Discussion

It has been shown that intact IgG is more effective in

FIGURE 17

EFFECT OF PASSIVE TRANSFER OF ANTIBODIES ON THE DEVELOPMENT OF IgM MEMORY

The direct PFC response of lethally irradiated mice which received 2 x 10^7 cells from primed donors (○), primed donors given various amounts of IgG (log_2 haemagglutinin titre 9) (●) or $F(ab')_2$ antibody (log_2 haemagglutinen titre 8) (▲) was assessed five days after cell transfer along with a challenge of 4 x 10^8 SRBC. The control response of mice which received an equal number of cells from normal donors (□) with the challenge was assayed at the same time. The primed donor mice received 1 x 10^6 SRBC thirty-seven days before sacrifice and the antibody injections were given one day after the priming antigen injection. Each point represents the arithmetic mean of the PFC per spleen of six recipient mice and the vertical lines represent one standard deviation of the reported mean.


PLAQUE-FORMING CELLS PER SPLEEN
10^5
10^4
10^3
0
0.003
0.03
0.3
ML OF ANTIBODY GIVEN TO THE CELL DONOR

suppressing the immune response than its $F(ab')_2$ fragment (Sinclair, 1969; Sinclair et al., 1970). Intact IgG inhibits IgG priming while its $F(ab')_2$ fragment does not have any effect on IgG priming (Sinclair, 1969). We have shown that, in intact animals, IgG suppresses IgM secondary response (Fig. 5). On transfer into irradiated hosts, however, cells from primed mice given IgG or $F(ab')_2$ antibody gave a normal IgM secondary response. This indicates that the development of IgM memory is taking place regardless of the presence of IgG or $F(ab')_2$ antibodies. It has been shown that in intact animals IgG injection just prior to or with the challenge antigen results in a marked suppression of IgM secondary response (Figs. 9 and 10). It seems that the expression of IgM memory upon antigenic challenge is suppressed in the presence of IgG antibodies. This is why transfer of spleen cells from primed IgG treated animals to an IgG free environment in the irradiated recipients results in the release from this suppression.

4. EFFECT OF PASSIVE IgG ANTIBODY ON IgM RESPONSE OF NORMAL AND PRIMED CELLS

Introduction

It has been shown earlier that passive IgG injection

within seconds or along with the challenge results in a suppressed IgM secondary response in the intact animals. This observation has been considered to mean that IgG antibodies inhibit the expression of IgM memory of the cells. It could however, be argued that one week after the priming antigen, other unknown factors such as migration of the cells to protected sites may have come into play in the intact animal. In the intact animals primed cells were shown to be more resistant to suppression by IgG compared to the normal cells. However, in this system, the amount of antibody in the primed animals was difficult to control because of the possibility of active antibody synthesis from the priming antigen. To prove that IgG antibodies in fact inhibit the cells from responding to antigenic challenge, and to compare the suppressive effect of IgG in primed and normal cells, it was important to isolate cells and show that they respond to challenge antigen in the absence of IgG but not in its presence. This was achieved by obtaining primed or non-primed cells from the donor spleens and transferring them into irradiated recipients with or without IgG antibodies.

Procedure

Mice were primed with a single injection of 5×10^5

SRBC. One week after priming, mice were sacrificed and 2×10^7 spleen cells transferred into groups of irradiated recipients. All groups of mice were given varying amounts of IgG antibody within thirty seconds of the cell transfer and challenged with 4×10^8 SRBC. Spleen cells from normal donors were transferred to other groups of irradiated recipients in the same way. Cells from primed and normal mice were transferred to other groups to serve as controls. One control group in primed cell recipients received 0.1 ml of normal mouse serum. Five days after the cell transfer, the recipients were killed and assayed for their immune response.

Results

In Fig. 18, it can be seen that an equal number of cells from primed donors produced ten times as many PFC as the cells from non-primed donors upon transfer into irradiated hosts. This difference in the response shows considerable amount of priming at one week. Normal mouse serum (0.1 ml) given to the cell recipients within seconds of cell transfer did not have any effect on the secondary IgM immune response of these cells. Specific IgG antibody given to the recipients in as small an amount as

0.001 ml, was sufficient to suppress significantly the primary and secondary immune response. An amount of antibody equivalent to 0.01 ml suppressed the primary response by one hundred-fold, and the same amount of antibody suppressed the secondary response given by the same number of cells by approximately twelve-fold. The degree of suppression of both primary and secondary response was directly proportional to the amount of antibody injected.

Discussion

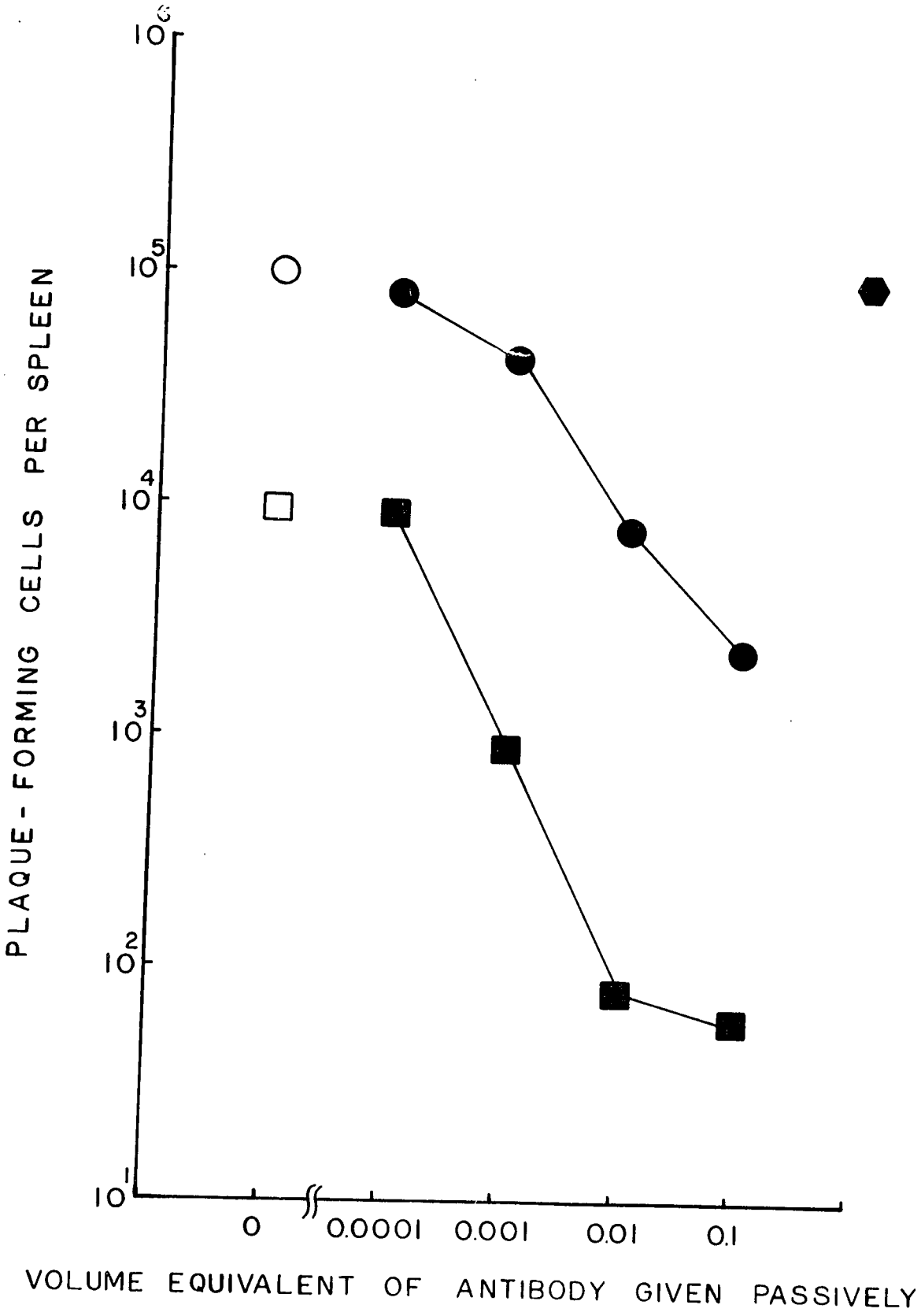
In the intact animals, a short interval after priming, the IgM memory starts to decline sharply (see Fig. 2). This decline may be due to the induction of a small IgG antibody response by the priming antigen. Transfer of cells from primed animals beyond this decline has been shown to result in a good secondary IgM response in the irradiated recipients (Fig. 16 and Cunningham (1969)).

However, it has not been shown if this release from suppression is due to the absence of IgG antibodies or due to some other factor(s). Our experiments show that in fact minute amounts of IgG can inhibit an IgM secondary response. The results therefore support the hypothesis that small amounts of IgG antibody, induced by priming antigen, may

FIGURE 18

SUPPRESSION OF IgM RESPONSE OF NORMAL AND PRIMED CELLS BY IgG ANTIBODIES

The direct PFC response of lethally irradiated mice which received 2×10^7 cells from normal (□) or primed (○) donors along with challenge antigen (4×10^8 SRBC) or challenge antigen and various amounts of IgG antibody (closed symbols) was assayed five days after the cell transfer. The control response of mice receiving an equal number of primed cells along with challenge antigen and 0.1 ml of normal mouse serum (●) was assessed at the same time. The primed mice received 5×10^5 SRBC eight days before sacrifice. The passive antibody given to the cell donors had a $\log_2$ haemolysin titre of 9. Each point represents the arithmetic mean of the PFC per spleen of six recipient mice.


PLAQUE-FORMING CELLS PER SPLEEN
10^6
10^5
10^4
10^3
10^2
10^1
0
0.0001
0.001
0.01
0.1
VOLUME EQUIVALENT OF ANTIBODY GIVEN PASSIVELY

be responsible for the decline in IgM memory in intact animals. The experiments also indicate that the IgM secondary response is less sensitive to suppression by IgG antibodies than is the IgM primary response. The reason for this resistance to suppression may be a larger proportion of immunocompetent cells in the primed cell-inoculum, compared to non-primed cell-inoculum. It is, however, possible that memory cells may be less sensitive to suppression by IgG than the virgin antigen-sensitive cell.

5. EFFECT OF ANTIGEN DOSE ON PRIMING

Introduction

A number of reports from various laboratories during the past decade have indicated that IgM memory does not exist (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964; Hege and Cole, 1966). Other reports indicate that it exists only for a short period (Wigzell, 1966). In most of these reports, large doses of antigen were used for priming. Later, Wigzell (1966) used small doses of antigen for priming to avoid inhibition of the IgM response by IgG antibodies. He could demonstrate considerable memory with this schedule. The failure of earlier workers to demonstrate IgM memory was therefore explained on the basis

of inhibition of the IgM response by IgG antibodies, produced as a result of large antigen doses.

As has been reported earlier in our dose-response experiments in the intact animals, a depressed secondary response was obtained when a large dose of antigen was used for priming. It was therefore of interest to find if cells from such animals would give an increased secondary response upon transfer into irradiated recipients. A number of experiments were performed to determine the priming antigen dose-response relationship in cell transfer experiments.

Procedure

Groups of mice were primed with antigen dose varied over a ten thousand-fold range (10^5 SRBC to 10^9 SRBC). Various times after priming, cells from each group of mice were prepared and transferred into irradiated recipients along with 4×10^8 SRBC for challenge. A control group receiving an equal number of normal cells with antigen was included. Five days after the cell transfer, all mice were killed and their spleens assayed for the PFC response.

Results

Figure 19a, b and c show the IgM response of lethally irradiated mice which received cells from mice primed with

varying antigen dose for one week, two weeks and eleven weeks respectively. It can be seen that, whether the cells from donor mice were transferred one, two, or eleven weeks after the priming antigen injection, the recipients gave similar patterns of response to the antigen challenge. Significant priming was established by the smallest antigen dose used (i.e., 1×10^5 SRBC). Whether the duration of priming was short or long, the peak of priming was obtained with 10^7 SRBC as the priming dose. At all times of priming, the use of a large dose of antigen (i.e., 1×10^9 SRBC) gave a significantly lower response than was obtained with a medium priming dose (i.e., 1×10^7 SRBC). However, as the duration of priming increased, the responsiveness of cells from donors primed with a large dose seemed to recover. A complete recovery of responsiveness of the cells from animals primed with a large dose was not seen during the eleven weeks after the priming injection. Fig. 20 shows the IgM and IgG response of mice which received cells from donors primed with varying antigen doses for one week prior to cell transfer. It can be seen that IgG response follows a pattern similar to the IgM response. The peak IgG response, however, occurs when the priming antigen dose is ten times larger than

that for peak IgM response. When 10^9 SRBC was used for priming, the IgG secondary response was also significantly suppressed.

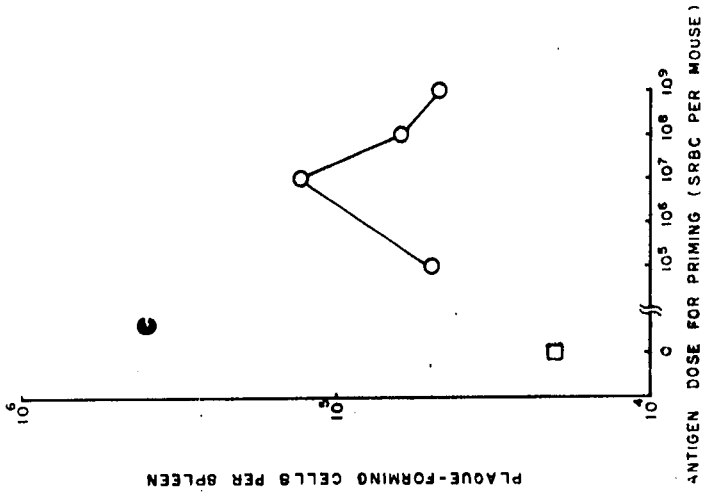
Discussion

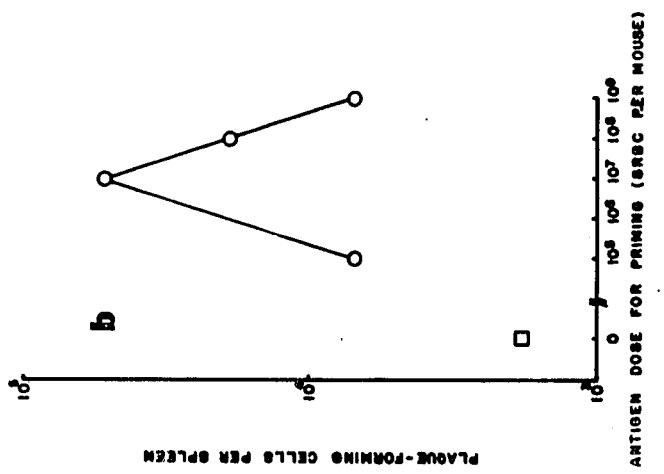
The depression of secondary response upon priming with a large dose of antigen was expected in intact animals. This expectation was based on the grounds that a larger dose of antigen produces large amounts of IgG antibodies which would inhibit IgM secondary response. The failure of these cells to recover responsiveness upon transfer into irradiated recipients was, however, unexpected. This inhibition of response in recipients was in contrast to the observation with cells from animals primed with 5×10^5 SRBC for a long duration. The results therefore indicated that this depressed response of the cells from animals primed with a large dose may have a mechanism other than antibody-mediated suppression. This view was supported by the observation that cells from donors primed and treated with antibody gave a normal secondary response in irradiated recipients (Fig. 17). These results, however, do not rule out the possibility that this suppression may actually involve IgG mediated immunosuppression for the following

FIGURE 19

EFFECT OF PRIMING ANTIGEN DOSE ON IgM PRIMING

The direct PFC responses of lethally irradiated recipients, receiving 2 x 10^7 cells from normal (□) or donors primed with various amounts of antigen (◯) were assessed five days after transfer of cells along with challenge antigen (4 x 10^8 SRBC). The primed donors received the priming antigen injection, one week (a) two weeks (b) or eleven weeks (c) before sacrifice. Each point represents arithmetic mean of the PFC per spleen of six recipient mice and the vertical lines represent one standard deviation of the reported mean.


PLAQUE-FORMING CELLS PER SPLEEN
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)


PLAQUE-FORMING CELLS PER SPLEEN
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

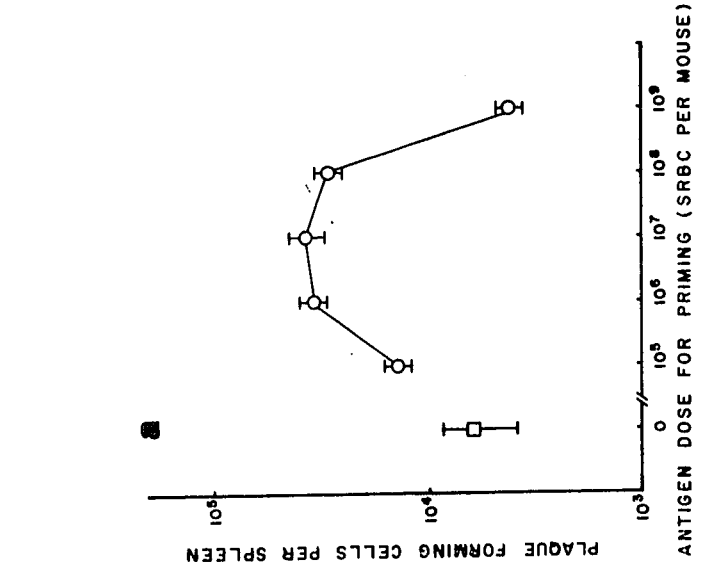
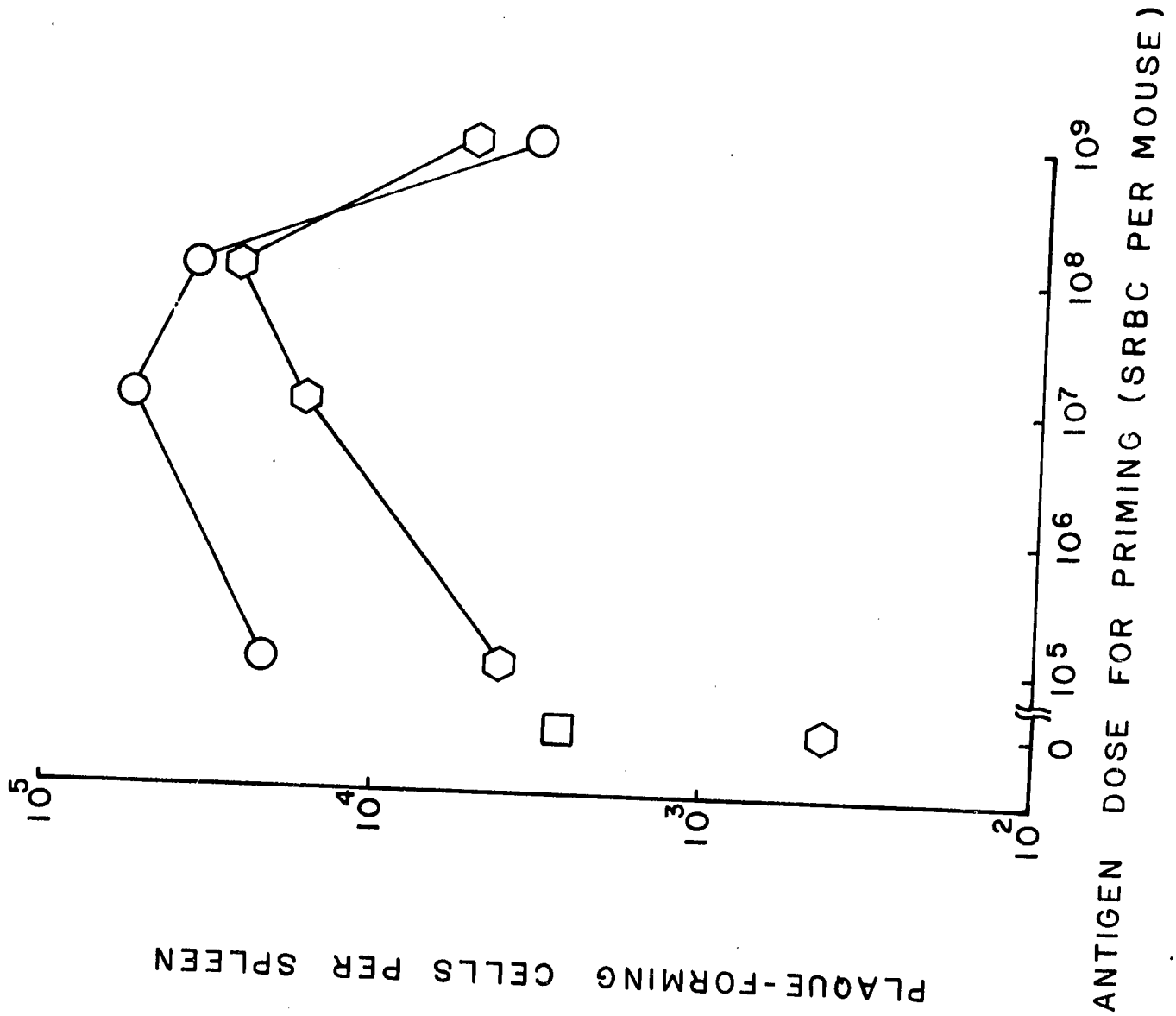

PLAQUE FORMING CELLS PER SPLEEN
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

FIGURE 20

SUPPRESSION OF IgM AND IgG PRIMING BY LARGE DOSES OF ANTIGEN

The direct (○) and indirect (⬡) PFC responses of lethally irradiated mice which received 2 x 10^7 cells from donor mice primed with various amounts of antigen were assessed five days after cell transfer along with challenge antigen (4 x 10^8 SRBC). The open square (□) indicates the control direct PFC response of mice receiving an equal number of cells from normal donors along with the challenge antigen, assayed at the same time as other groups. Each point represents the arithmetic mean of the PFC per spleen of six recipient mice. The primed donors were given the priming antigen injection nine days before sacrifice.


PLAQUE-FORMING CELLS PER SPLEEN
10^5
10^4
10^3
10^2
0
10^5
10^6
10^7
10^8
10^9
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

reasons:

1) It has been shown that a large dose of antigen at one week results in an extensive IgG response. In spite of the fact that the cells were washed a number of times, there may still be sufficient amount of IgG antibodies carried over along with the cells.

2) There is a possibility that IgG-producing cells transferred in the inoculum may have caused this inhibition of the secondary immune response.

3) Antigen-antibody complexes, carried on or inside the cells may be responsible for the suppressed secondary response in the recipients of large dose primed cells.

It was therefore desirable to perform more experiments, designed to elucidate the mechanism of the suppression of the immune response induced by priming with large doses of antigen.

6. EFFECT OF NUMBER OF WASHES ON THE IMMUNE RESPONSE OF CELLS FROM DONORS PRIMED WITH A LARGE DOSE OF ANTIGEN

Introduction

It has been mentioned earlier that the possibility exists that spleen cells derived from animals which received a large dose of antigen for priming may contain some IgG antibody. In spite of usual washing procedures, some of this antibody may be carried over along with the

cells. This antibody would then be able to inhibit the secondary response in the cell recipients. If free antibody is carried over in the cell inoculum, it should be possible to remove a larger amount of this antibody if the cells are washed a greater number of times. Therefore, one should be able to get a greater response from large dose primed cells when they are washed a greater number of times, and a smaller response if the cells are washed a few times only. Based on this rationale, the following experiment was performed.

Procedure

Mice were primed with small (10^5 SRBC), medium (10^7 SRBC) or large (10^9 SRBC) antigen doses. Eight days later the mice were sacrificed and cell suspensions prepared from their spleens. At the same time cells from the spleens of normal mice were also prepared. The cells from non-primed, 10^5 SRBC and 10^7 SRBC primed donors were washed twice in large volume of ice cold Medium 199. The cells from 10^9 SRBC primed donors were either not washed at all, or washed one to four times in large volumes of Medium 199. Groups of irradiated mice received 2×10^7 cells along with the challenge antigen (4×10^8 SRBC). Five

days after the cell transfer, mice were bled and sacrificed and their immune response assayed.

Results

Table I shows the results of the experiment. It can be seen that cells from donors primed with a medium dose of antigen gave a response which was about eight-fold higher than that given by cells from non-primed donors. Moreover, the response given by cells from large dose primed donors was markedly suppressed, compared to the immune response given by cells derived from medium dose primed donors, in spite of a number of washes. It can also be seen that whether the cells from donors primed with a large dose of antigen were washed four times before transfer, or were not washed at all, the response was similar.

Discussion

There was no correlation between the number of washes that the large dose primed cells received and their immune response. The results indicate that whatever factor or factors are involved in the suppression of the immune response of the cells derived from animals primed with a large dose of antigen, they can not be removed by washing the cells. This still leaves the possibility that either the

TABLE I

EFFECT OF IN VITRO WASHES ON THE IMMUNE RESPONSE OF CELLS DERIVED FROM DONORS PRIMED WITH A LARGE DOSE OF ANTIGEN

Cell donors primed with*	Number of times cells washed before transfer	**PFC per recipient spleen
-	2	7,135 ± 1,611
10^5 SRBC	2	33,365 ± 4,956
10^7 SRBC	2	51,470 ± 4,409
10^9 SRBC	-	5,675 ± 1,403
10^9 SRBC	1	7,575 ± 1,321
10^9 SRBC	2	9,500 ± 2,784
10^9 SRBC	3	3,900 ± 453
10^9 SRBC	4	8,455 ± 847

* Donor mice were given an intravenous injection of the amount of antigen indicated one week before their cells were transferred to the recipients.

**Each value represents the arithmetic mean of the PFC per spleen ± the standard error, of six mice, five days after transfer of 2 x 10^7 spleen cells along with challenge (4 x 10^8 SRBC).

suppressive antibody is carried on or inside the cells or is produced by some cells present in the inoculum, inside the host.

7. EFFECT OF PASSIVE TRANSFER OF IgG ANTIBODIES UPON THE RESPONSIVENESS OF CELLS FROM ANIMALS PRIMED WITH A LARGE DOSE OF ANTIGEN

Introduction

It is well known that IgG antibodies inhibit both IgG and IgM response. Moreover, IgG antibodies are known to inhibit IgG priming, while IgM priming is less sensitive to inhibition by IgG antibodies (Sinclair and Chan, 1971). It was hypothesized that, if the suppressed response of large dose primed cells in the recipients is due to the presence of IgG antibody-forming cells in the inoculum, then it would be possible to suppress these cells by passive transfer of IgG antibodies to the cell donors. Under this condition, the large dose primed cells should respond like medium dose primed cells. If the response is suppressed because the amount of antigen given for challenge is less than that used for priming (Nossal et al., 1965), a larger challenge dose would result in a good secondary response.

Procedure

Mice were injected with either 1×10^5, 1×10^7 or 1×10^9 SRBC. One day after the antigen, some of the mice from each group were given 0.1 ml of anti-SRBC IgG antibodies. Seven days after the injection of the antibody, all mice were killed and cell suspensions prepared from their spleens. An equal number of cells were transferred into irradiated recipients, each group receiving 2×10^7 cells per mouse from donors which received one type of treatment. Antigenic challenge with 4×10^8 SRBC was given to all recipients at the time of cell transfer. One group of irradiated mice received spleen cells from normal donors, along with an antigenic challenge with 4×10^8 SRBC. Another group of mice which received cells from animals primed with 10^9 SRBC was given an antigenic challenge of 4×10^9 SRBC. Five days after the cell transfer, all mice were bled and their spleens assayed for PFC response.

Results

It can be seen in Fig. 21 that the largest secondary response was obtained with cells from mice primed with 1×10^7 SRBC. These recipients gave a response which was

fifty-fold greater than the primary response. It can also be seen that mice which received cells from donors primed with 10^9 SRBC gave a response which was only two and one-half times larger than the primary response. Therefore, there was a twenty-fold decrease in the response of large dose (10^9 SRBC) primed cells compared to that of medium dose (10^7 SRBC) primed cells. Increasing the challenge antigen dose resulted in a further decrease in the response of these cells, so that when ten times more antigen was given for challenge, the response was not different from the primary response. It can also be noted that, when the donors were given a passive injection of IgG antibodies one day after the priming antigen, the responses of small and large dose primed cells were increased. The response of cells from animals primed with a large dose was increased by the injection of passive IgG antibody to the donors to the extent that it was not different from the response of the medium dose primed cells. The low dose (10^5 SRBC) primed cells also responded with an increased immune response, when the donors were treated with IgG antibodies one day after priming. This increase was, however, smaller in degree, and was not obtained consistently.

Similar results obtained from another experiment performed in identical way are shown in Figure 22. In this

experiment, it can also be seen that if cells from large dose (10^9 SRBC) primed donors were transferred into hosts, and no antigenic challenge was given, they did not give any significant response.

Discussion

The results show that the suppression of the IgM response is not due to the challenge dose being smaller than the priming dose. The results also show that administration of IgG antibody to the donors, primed with a large dose of antigen, results in an increased adoptive immune response by the cells derived from such donors. It does not, however, mean that this increased responsiveness is due to the suppression of IgG-forming cells by passive IgG given to the donors. The reason is that IgG given passively would not only suppress IgG response, but would also neutralize some of the antigen, and perhaps remove it from the circulation. This may be equivalent to reducing the effective antigen. To test this possibility, $F(ab')_2$ was used which can combine with the antigen, but does not suppress the immune response.

FIGURE 21

THE EFFECT OF LARGE DOSE FOR CHALLENGE ON THE IgM RESPONSE OR PRIMED CELLS

The direct PFC response of lethally irradiated mice receiving 2×10^7 cells from donors injected with various amounts of antigen (○) or antigen and 0.1 ml of IgG antibodies (●) were assayed five days after cell transfer and challenge with 4×10^8 SRBC. Control response of mice which received an equal number of cells from normal donors along with the challenge (□) is indicated. The response of a group of mice which received 2×10^7 cells from donors primed with 10^9 SRBC along with a challenge with 4×10^9 SRBC (⬢) was also assessed five days after cell transfer. The donor mice received the priming antigen eight days before sacrifice. Each point represents the arithmetic mean of the PFC per spleen of six recipient mice.

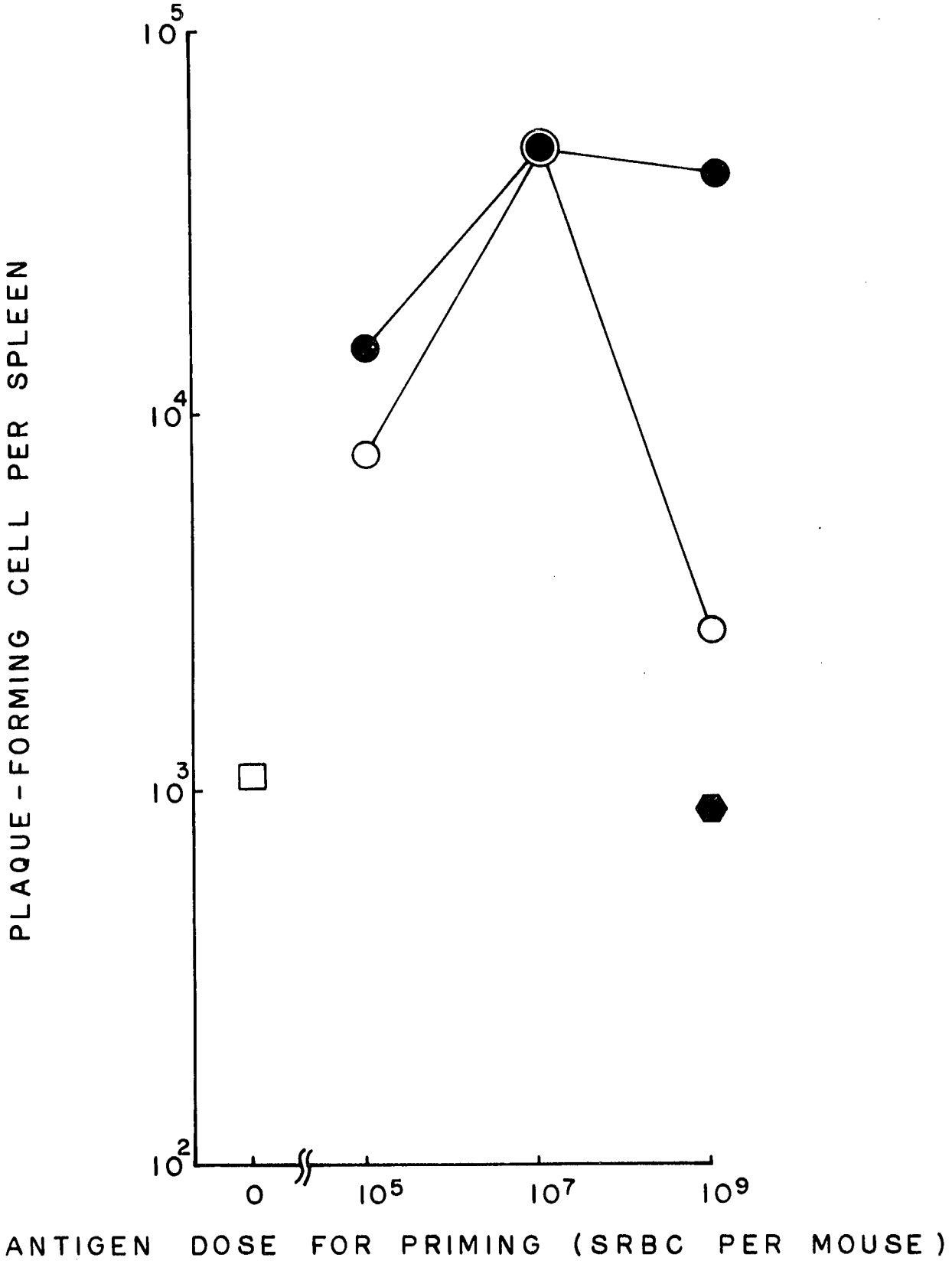
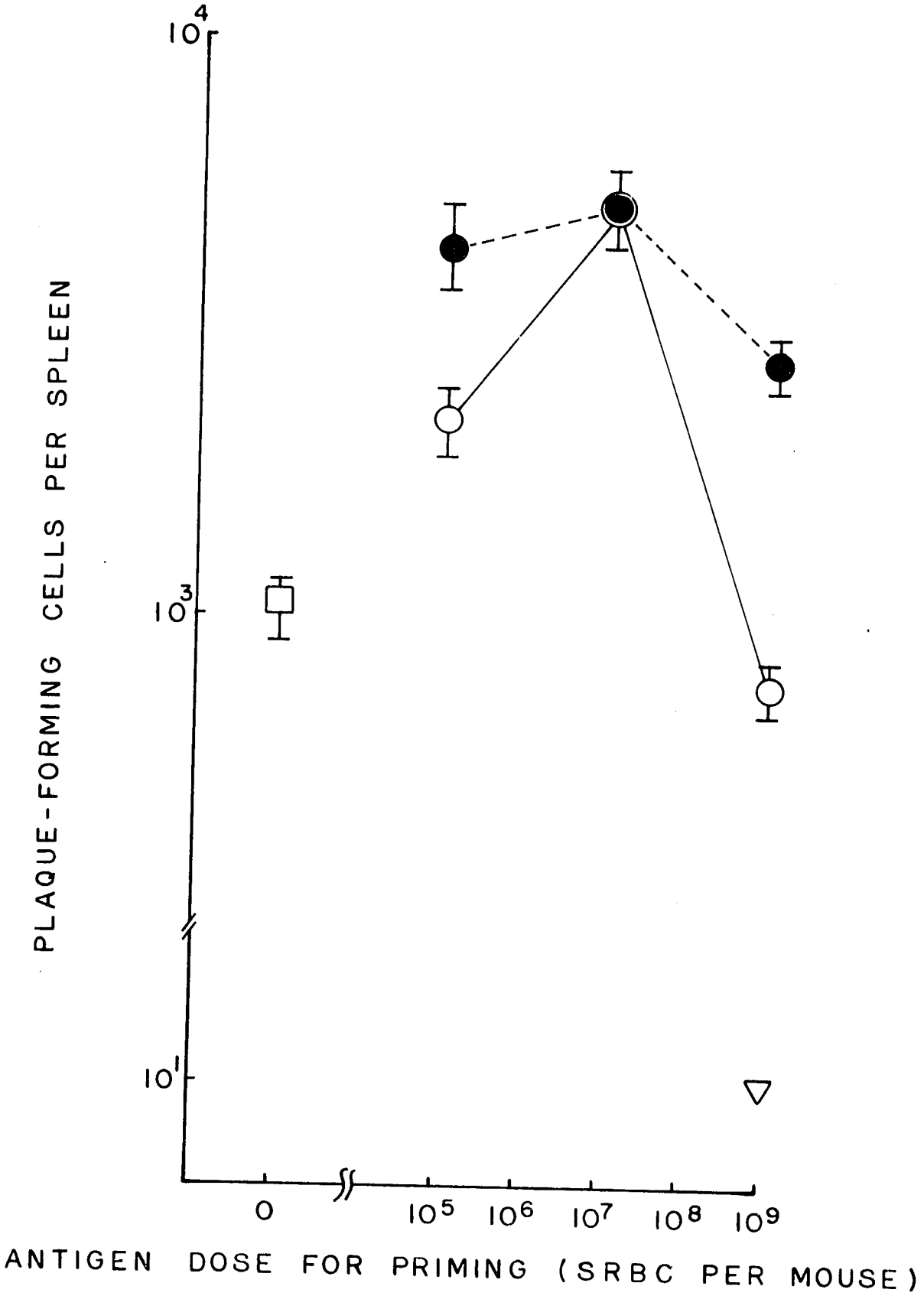

PLAQUE-FORMING CELL PER SPLEEN
10^5
10^4
10^3
10^2
0
10^5
10^7
10^9
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

FIGURE 22

THE EFFECT OF PASSIVE TRANSFER OF IgG ANTIBODIES TO THE PRIMED DONORS ON THE IgM RESPONSE GIVEN BY THEIR CELLS

The direct PFC responses of lethally irradiated mice receiving 2×10^7 cells from donors injected with various amounts of antigen (○) or antigen and 0.1 ml of IgG antibodies (●) were assessed five days after cell transfer and challenge with 4×10^8 SRBC. The control response of mice receiving an equal number of cells from normal donors (□) five days after the challenge is also indicated. The response of mice which received an equal number of cells from donors primed with 10^9 SRBC but did not receive a challenge (▽) was assayed at the same time. The primed donors received the priming antigen eight days before sacrifice. The passive antibody injections were given one day after the priming antigen. Each point represents the arithmetic mean of the PFC per spleen of six mice and the vertical lines represent one standard deviation of the reported mean.


PLAQUE-FORMING CELLS PER SPLEEN
10^4
10^3
10^1
0
10^5
10^6
10^7
10^8
10^9
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

8. THE RELEASE FROM INHIBITION OF IgM RESPONSE OF CELLS IN IRRADIATED RECIPIENTS BY PASSIVE IgG ANTIBODY BUT NOT BY $F(ab')_2$ ANTIBODY GIVEN TO THE DONORS PRIMED WITH A LARGE DOSE OF ANTIGEN

Introduction

When a large dose of antigen (1×10^9 SRBC) is used to prime the cell donors, the cells from such donors give a poor secondary immune response in the irradiated recipients. It was thought that this reduced responsiveness could be due to two possible reasons: (1) Induction of an IgG response by the priming antigen dose, which would suppress the IgM response because a large number of IgG forming cells are transferred in the cell inoculum. (2) Exhaustive differentiation of the memory cells to antibody-forming cells in the donor, according to the X-Y-Z scheme of Sercarz and Coons (1962), Byers and Sercarz (1968). To test the possibility that IgG antibody-forming cells were responsible for this inhibition, an experiment was designed on the following rationale. If IgG antibody formation in the donor could be inhibited, then cells from large dose primed donors should recover their ability to respond to

the antigenic challenge, in the recipient. Passive injection of IgG antibody inhibits IgG response as well as IgG priming, while it does not affect IgM priming, and $F(ab')_2$ fragment of IgG antibody is extremely poor in this respect (Sinclair, 1969; Sinclair et al., 1968, 1970). It was expected that if the response of the cells from large dose primed donors was suppressed in the recipients, due to IgG antibody-forming cells in the inoculum, it would be restored by passive IgG injection but not by its $F(ab')_2$ to the cell donors.

Procedure

Groups of mice were given a single intravenous injection of either 1×10^7 or 1×10^9 SRBC. One day after the SRBC injection, some of the mice which received the large dose of antigen were injected with varying amounts of IgG antibody to SRBC. Other mice which received the large dose of antigen were injected with varying amounts of $F(ab')_2$ prepared from the IgG antibody. Other mice were given neither IgG nor $F(ab')_2$ antibody. Both the IgG and the $F(ab')_2$ were equal in their antigen-binding activity as measured by haemagglutination technique. Eight days after the antigen injection, all mice were sacrificed and cell

suspensions prepared from their spleens. After several washings 2 x 10^7 cells per mouse were transferred to groups of irradiated mice. Each group of mice received cells from donors given one type of treatment. One group of mice received an equal number of cells from normal mice and served as a control primary response. At the time of cell transfer, all groups of mice received a challenge with 4 x 10^8 SRBC. Five days after the cell transfer, the recipients were sacrificed and their spleens assayed for PFC response.

Results

As can be seen in Fig. 23, transfer of 2 x 10^7 cells from donors which received a medium dose of antigen (1 x 10^7 SRBC) resulted in a response in recipients, which was more than twenty-fold larger than the response given by the same number of non-primed cells. Furthermore, if the donors were primed with a large antigen dose (1 x 10^9 SRBC) the same number of cells gave a response which was about one-tenth of the response given by medium dose primed cells. Cells from donors which received IgG antibody in addition to the large dose of antigen gave a response which was nearly as large as that given by medium dose primed cells. The response of the cells from the donors which received

0.01 or 0.1 ml of $F(ab')_2$ antibody in addition to the large dose of antigen was not different from the response of cells derived from donors which received the large dose of antigen only. The cells from donors receiving 1.0 ml of $F(ab')_2$ gave an increased response, but were still less responsive than those which received 0.01 ml of undigested IgG.

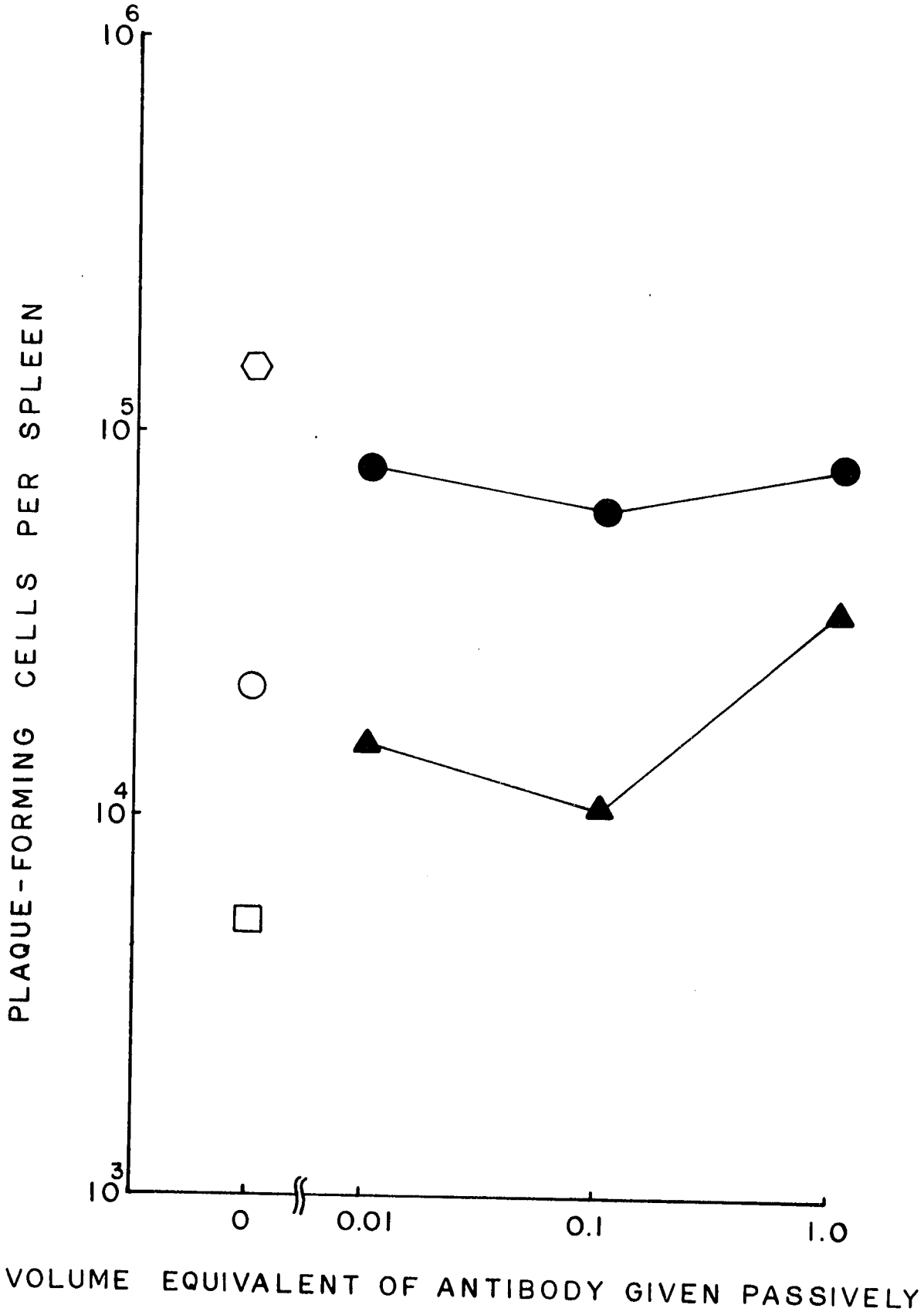
Discussion

It is established that IgG suppresses IgG primary response as well as IgG priming (Sinclair, 1969; Uhr and Baumann, 1961; Wigzell, 1967). $F(ab')_2$ fragment of IgG is very poor in its ability to suppress both IgM and IgG immune response (Sinclair, 1969; Sinclair *et al.*, 1968, 1970) while it is not different from IgG in its capacity to bind to antigen, and presumably cover antigenic determinants. Since the use of large doses of antigen for priming results in a depressed response, it has been claimed that this loss of memory is due to exhaustive differentiation of memory cells to antibody-forming cells (Sercarz and Byers, 1967; Byers and Sercarz, 1968; Hanna and Peters, 1971; Hanna *et al.*, 1969) upon repeated antigenic stimulation. The differential activity of $F(ab')_2$ and IgG anti-

FIGURE 23

THE INCREASED IgM RESPONSE GIVEN BY PRIMED CELLS ON TREATMENT OF THE DONORS BY IgG BUT NOT BY $F(ab')_2$ ANTIBODY

The direct PFC responses of lethally irradiated mice receiving 2 x 10^7 cells from mice primed with 10^9 SRBC (○) and injected with various amounts of IgG (●) or $F(ab')_2$ antibodies (▲) ($\log_2$ haemagglutinin titre 7.0) were assessed five days after cell transfer and challenge with 4 x 10^8 SRBC. Control responses of mice receiving an equal number of cells from normal mice (□) or mice primed with 10^7 SRBC (◯), along with the challenge were also assessed at the same time. The donor mice received the priming antigen eight days before sacrifice. Each point represents the arithmetic mean of the PFC per spleen of six recipient mice.


PLAQUE-FORMING CELLS PER SPLEEN
10^6
10^5
10^4
10^3
0
0.01
0.1
1.0
VOLUME EQUIVALENT OF ANTIBODY GIVEN PASSIVELY

body in the suppression of IgG response, while possessing the same capacity to bind to the antigen, was utilized in order to distinguish antibody-mediated suppression and memory cell exhaustion upon large dose priming. If it is the repeated antigen contact which results in the exhaustion of memory, by forcing the memory cells to irreversibly mature to antibody-forming cells, then binding of the antigen by either IgG or $F(ab')_2$ should preserve memory by removing the stimulus. However if $F(ab')_2$ binds to the antigen but does not remove the antigenic stimulus or intact IgG inhibits the differentiation of memory cells to antibody-forming cells but $F(ab')_2$ is deficient in this respect, then similar results would be obtained. The results indicate that the rescue of IgM memory by IgG antibody may be either due to IgG antibody-mediated suppression of IgG response in the recipient or suppression of the differentiation of memory cells to antibody-forming cells in the donors. A slight restoration of the responsiveness by the passive transfer of a large amount of $F(ab')_2$ may be explained on the basis of some innate immunosuppressive activity in the $F(ab')_2$ antibody. Alternatively minor contamination of $F(ab')_2$ with intact IgG antibody during isolation of $F(ab')_2$ from IgG antibody may explain this result (Sinclair, 1969;

Sinclair et al., 1968, 1970).

9. IN VIVO SUPPRESSION OF IgM SECONDARY RESPONSE BY CELLS FROM DONORS PRIMED WITH A LARGE DOSE OF ANTIGEN

Introduction

The cell washing experiment indicated that there is very little, if any, free IgG which is transferred with the cells from large dose primed donors. The restoration of the immune response of cells from donors primed with a large antigen dose by passive IgG, but not by $F(ab')_2$ antibody, indicated that the suppressed response of large dose primed cells may be due to antibody mediated suppression. Since antibody which could be washed off the large dose primed cells is not present in the inoculum, two possibilities remain which could explain the suppressed response of these cells: (a) IgG antibody-forming cells present in the inoculum produce sufficient amounts of suppressive antibody in the recipient. (b) Antigen-antibody complexes tightly bound to the cells are transferred with the cells. These complexes may be released from the cells in the recipient and either dissociate providing free antibody or may themselves be immunosuppressive.

Whatever the mechanism of immunosuppression in large dose primed cells, if antibody or antigen-antibody complexes are responsible for this, then cells from donors primed with a large dose of antigen should be able to suppress the immune response of other immunocompetent cells, when transferred along with them. If, on the other hand, the suppressed immune response of cells derived from donors primed with a large dose of antigen is due to the absence of memory cells, then these cells should have no effect on other immunocompetent cells.

Procedure

Mice were primed with either a medium dose (1×10^7 SRBC) or large antigen dose (1×10^9 SRBC). Eight days later mice were sacrificed and spleen cells from each group prepared. At the same time, spleen cells from untreated mice were also similarly prepared. Irradiated mice were divided into several groups. One group of mice each received 1×10^7 cells per mouse from either non-primed, medium dose primed or large dose primed donors. Similarly, one group of mice each received 2×10^7 cells per mouse from either non-primed, medium dose primed, or large dose primed donors. One group of mice received

10^7 cells from donors primed with the medium dose of antigen, and 10^7 irradiated (1500 rads) cells from donors primed with the large dose of antigen. Another group of mice received 2×10^7 cells from donors primed with a medium dose of antigen and 2×10^7 cells from donors primed with a large dose of antigen. A third group received 2×10^7 cells from donors primed with a medium dose of antigen and 2×10^7 irradiated (1500 rads) cells derived from donors primed with a large dose of antigen. A control group of mice received 2×10^7 irradiated (1500 rads) cells derived from donors primed with a large dose of antigen. All groups of mice were given 4×10^8 SRBC as challenge antigen. Five days after the cell transfer and challenge, all mice were sacrificed and their spleens assayed for PFC response.

Results

It can be seen in Table II that 1×10^7 medium dose primed cells gave a PFC response about twenty times larger than the response given by an equal number of non-primed cells. The response given by an equal number of large dose primed cells was not different from that of the non-primed cells. Similarly 2×10^7 medium dose primed cells gave a response about ten times larger than that given by an

equal number of non-primed or large dose primed cells. Moreover, it can be seen that 2 x 10^7 large dose primed cells when given 1500 rads of γ-radiation, and then transferred into recipients along with the antigen did not give a significant immune response. The response given by these cells was only one-thousandth that of the response given by an equal number of non-primed cells, or large dose primed cells not irradiated before transfer. When 10^7 medium dose primed cells were mixed with an equal number of large dose primed irradiated cells and transferred into irradiated hosts, they gave an immune response which was less than one-fourth of that given by 10^7 medium dose primed cells alone. When 2 x 10^7 cells derived from donors primed with a medium dose of antigen were transferred along with 2 x 10^7 cells derived from donors primed with a large dose of antigen, they gave a response which was less than the response given by 10^7 medium dose primed cells alone and was less than one-half of the response given by 2 x 10^7 medium dose primed cells alone. Similarly 2 x 10^7 large dose primed irradiated (1500 rads) cells were able to suppress the immune response of 2 x 10^7 medium dose primed cells to less than one-

half.

Discussion

It has been suggested that the suppressed secondary response upon priming with a large dose of antigen is due to the exhaustive differentiation of immunocompetent cells with the resultant depletion of Y-cell compartment (Sercarz and Coons, 1962; Byers and Sercarz, 1968; Hanna and Peters, 1971; Hanna et al., 1969). It has earlier been shown that this inhibition can be prevented by IgG antibody injection but not by $F(ab')_2$ antibody injection to the cell donors. It has also been shown that washing the cells a number of times before transfer does not have any significant effect on this inhibition. It has thus been shown that this suppression is most probably due to IgG, but that this IgG can not easily be washed off the cells. This IgG however, may exist in the donor inoculum as antigen-antibody complexes. The results of this experiment are consistent with the previous findings in other experiments. The suppression of the response of medium

TABLE II

SUPPRESSION OF THE PFC RESPONSE OF CELLS FROM DONORS PRIMED WITH MEDIUM DOSE OF ANTIGEN BY THE CELLS FROM MICE PRIMED WITH A LARGE DOSE

Cell donors primed with			[1] PFC per recipient spleen
-	10^7 SRBC	10^9 SRBC	
10^7 [2]	-	-	2,513 ± 1,001
2×10^7	-	-	9,573 ± 3,707
-	10^7	-	62,750 ±16,453
-	2×10^7	-	88,833 ± 9,787
-	-	10^7	1,606 ± 326
-	-	2×10^7	8,916 ± 1,508
-	-	2×10^7 (IRR)	10 ± 3
-	10^7	10^7 (IRR)	12,616 ± 2,949
-	2×10^7	2×10^7 (IRR)	40,583 ± 3,911
-	2×10^7	2×10^7	39,950 ± 7,225

[1] Each value represents the arithmetic mean ± the standard error of PFC obtained in six mice, five days after cell transfer and challenge with 4×10^8 SRBC.

[2] Number of cells that were transferred to each recipient mouse (IRR) Cells were given 1500 rads of γ-radiation *in vitro* before transfer.

dose primed cells by large dose primed cells therefore could be achieved by the secretion of IgG antibody by the IgG antibody-forming cells present among the large dose primed cells. However, the results do not exclude the possibility that this may be due to release of antigen-antibody complexes by the cells.

10. EFFECT OF IN VITRO INCUBATION OF CELLS ON THEIR IMMUNE RESPONSE IN LETHALLY IRRADIATED RECIPIENTS

Introduction

It has been shown that cells derived from animals primed with a large dose of antigen (10^9 SRBC) gave a response which was markedly depressed compared to the immune response given by the cells derived from animals primed with a medium dose (10^7 SRBC). It has further been shown that this suppression can not be removed if the cells are given a number of washes before transfer

into the irradiated recipients. A release from suppression can however be affected if the cell donors are given passive IgG antibody one day after the antigen but not with $F(ab')_2$ antibody given at this time. These observations have suggested that either IgG producing cells, transferred in the cell inoculum are responsible for this suppression by virtue of IgG production in the recipients, or this suppression is affected by antigen-antibody complexed, carried over with the cells. It has been reported that antigen, bound to the cells from primed donors, can be released by incubation of the cells at 37°C in vitro (Mitchison, 1969). If the suppressed response of cells derived from the animals primed with 10^9 SRBC is due to antigen-antibody complexes, incubation of such cells in vitro might result in the dissociation of antigen from the cells and their consequent release from inhibition. On this rationale, the following experiment was performed.

Procedure

Mice were given a single intravenous injection of 10^7 or 10^9 SRBC or were given no treatment. Eight days after the antigen injection the mice were sacrificed and spleen cell suspensions prepared from them. The spleen

cell suspensions from mice receiving each type of treatment were washed three times in large volumes of Medium 199 and resuspended in CMRL 1066 (GIBCO) with 10% foetal calf serum. The cells were divided into two parts. One-half of the cells from each type of donors was incubated at 37°C while the other half was kept cool in ice. After two hours of incubation, all cells were again washed three times in Medium 199. Groups of irradiated mice were injected with 2×10^7 cells which had received one type of treatment. In addition, all groups of recipients were given 4×10^8 SRBC as challenge at the time of cell transfer. Five days after the cell transfer, the recipients were killed and their immune response was assayed.

Results

In Figure 24, it can be seen that mice which received cells from donors injected with 10^7 SRBC, gave a secondary response which was considerably higher than the response given by mice which received cells from normal donors. The response given by mice which received cells from donors injected with 10^9 SRBC for priming, was not different from control primary response. Furthermore, it can be seen that incubation at 37°C for two hours before transfer into re-

cipients did not have any effect on the immune response of cells derived from donors primed with either 10^7 or 10^9 SRBC. The immune response of the animals which received cells from normal donors, incubated at 37°C before transfer into recipients, was slightly higher than the immune response of those recipients which were given the cell preparation kept cold in ice.

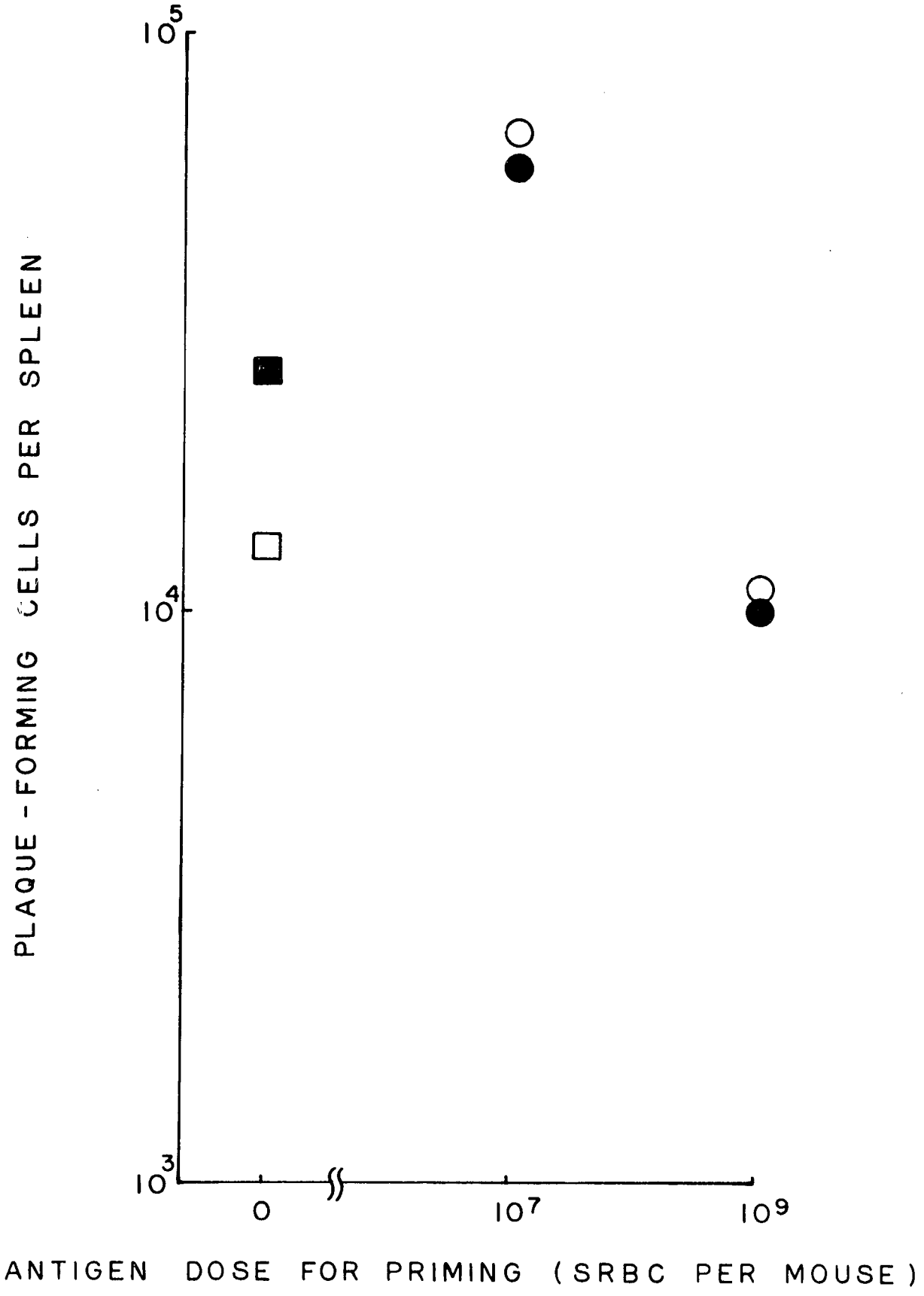
Discussion

It is obvious that incubation of the cells at 37°C did not have any effect on the immune response of cells derived from donors which were given either 10^7 or 10^9 SRBC. The slight increase in the immune response of cells derived from normal mice, which were incubated at 37°C, may be due to the stimulatory effect of the foetal calf serum on these cells. The lack of release from suppression of the cells from donors primed with 10^9 SRBC upon incubation at 37°C suggests that either antigen-antibody complexes are not involved in this suppression or that perhaps because we have used an antigen different from Mitchison (1969), the dissociation of antigen does not take place in this system.

FIGURE 24

EFFECT OF IN VITRO INCUBATION ON THE IgM RESPONSE OF CELLS IN RECIPIENTS

The direct PFC response of lethally irradiated mice which received 2 x 10^7 cells from donors primed with various amounts of antigen were assessed five days after cell transfer along with challenge dose (4 x 10^8 SRBC). The cells were either incubated at 37°C (closed symbols) or in ice (open symbols) for two hours before transfer to the recipients. Each point represents the arithmetic mean of the PFC per spleen of six recipient mice.


10^5
10^4
10^3
PLAQUE-FORMING CELLS PER SPLEEN
0
10^7
10^9
ANTIGEN DOSE FOR PRIMING (SRBC PER MOUSE)

V. GENERAL DISCUSSION

In the last decade, attempts by many laboratories to show the existence of memory for IgM class of antibodies have been unsuccessful (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964; Bauer et al., 1963), with a few exceptions (Nossal et al., 1965; Wigzell, 1966). This failure to demonstrate IgM immunological memory is in contrast to the well documented existence of IgG memory (Svehag and Mandel, 1964; Gottlieb et al., 1964; Uhr and Baumann, 1961a; Uhr, 1964; Bauer et al., 1963).

To demonstrate IgM memory, the time of assay after the challenge is extremely important. This point is borne out clearly from the time course of the primary and secondary immune response. This is due to the fact that, soon after the peak, there is a rapid decline in the antibody response of primed or non-primed animals. In addition to this, the peak of secondary response occurs approximately one day before the peak of the primary response. Therefore at a time when the secondary response is declining, the primary response is still increasing. Hence, it is important to assay the immune response at the

peak of secondary response, or just prior to this peak at a time when both the primary and secondary responses are increasing, in order to distinguish between a primary and a secondary response. The failure of many laboratories to show a significant IgM memory may have been due to assays performed at a time after the peak secondary response when significant difference between a primary and a secondary response could not be shown.

More recently, interest has been renewed in the study of IgM memory. A number of reports have recently appeared which indicate that IgM memory exists, though for a shorter duration (Wigzell, 1966; Hajek, 1970). It has been clearly shown that the selection of antigen dose for priming has an important bearing on the outcome of the challenge (Section IV, Figs. 13 and 14). Hajek (1970) using ØX174 in small doses, could show the establishment of IgM priming, while Uhr and Finkelstein (1963), using large doses of the same phage, had failed to achieve any priming. In contrast to our results, however, the maximum IgM memory in Hajek's (1970) experience is present about one month after the priming, while in our experience the peak of memory is arrived at about one week after priming. This apparent discrepency may originate due to some important differences in the ex-

perimental set-up. We have used mice as the experimental animals while Hajek (1970) has used rabbits. The antigen used by us is different from what Hajek has used. An important reason for the late arrival of the peak of memory in Hajek's (1970) experiments may be the age of the animals. He has used infant rabbits, in order to avoid production of IgG antibodies which may be formed in older animals and influence IgM memory. It is, however, known that the immune response matures with age, being optimum in adults (Sinclair and Millican, 1967; Rowley and Fitch, 1965; Makinodan and Paterson, 1966).

The short life of IgM memory claimed by Hajek (1970) and Wigzell (1966) may not be true, if memory cells become functionally blocked some time after priming. It has been shown by Cunningham (1969) that, whereas in the intact animals IgM memory declines rapidly, cells from such donors give a good secondary response when transferred even several months after priming. This finding supports the idea that in intact animals, the memory cells become functionally blocked at later intervals.

It is established that IgG antibodies inhibit the IgM response (Sahiar and Schwartz, 1964a; Moller and Wigzell, 1965; Uhr and Baumann, 1961). The results obtained in

this study support these findings (Section IV, Figs. 10 and 18). It is possible that IgG antibodies produced as a result of priming antigen are responsible for the inhibition of the IgM secondary response. It has been demonstrated by Cunningham (1969), as has already been mentioned, that transfer of cells from donors several months after priming, results in a good secondary response in irradiated recipients. Similar results have been obtained in this study (Section IV, Fig. 16). It has been shown that, while IgM memory declines rapidly in the intact animals, it does not appreciably decline on cell transfer. That the secondary response does not show a marked decline upon cell transfer, supports the view that IgG antibodies produced in the donors result in the suppression of secondary response upon antigenic challenge at longer durations.

It has been reported that passively transferred serum antibodies result in the suppression of immune response, if given with the antigen, or several days after the antigen (Moller and Wigzell, 1965; Uhr and Baumann, 1961). Moreover, Moller and Wigzell (1965) have shown that the inhibitory effect of passive antibody becomes apparent after a lag of twenty-four to forty-eight hours. This indicates that passive antibody does not inhibit antibody-forming

cells, but inhibits some step in the induction of antibody formation. It has been suggested that antibody operates in the suppression of immune response, through the binding of the antigen (Uhr and Baumann, 1961; Uhr, 1964). Accordingly, binding of the antigen by antibody could result in rapid degradation and removal of the antigen by phagocytosis. This would prevent the antigen from reaching the sites essential for the induction of antibody synthesis. The antibody, by binding to the antigen, could prevent interaction between antigenic determinants and the receptors on the antigen sensitive cells. This would, in turn, prevent the induction of antibody synthesis. That the first possibility is unlikely has been shown by Cerottini et al. (1969). In their experiments, antibody against one antigenic determinant resulted in suppression of antibody synthesis against that determinant, without affecting the antibody synthesis against a second determinant present on the same molecule. If destructive phagocytosis had been responsible for the suppressed response against one determinant, one would expect the suppression of immune response against both determinants, since the whole molecule would have been destroyed.

The effect of IgG antibodies on the secondary IgM immune potential of an animal, when the antibodies are given with or soon after the priming antigen, has not been clearly worked out. It was of interest, therefore, to study this effect, especially when small doses of antigen were employed. This was done in order to minimize the contribution of active antibody synthesis towards suppression, and to achieve antibody excess when the effects of passive antibody were studied. It has been shown that in intact animals IgG antibody given passively to the animals one to ten days after the priming antigen results in a suppressed immune response upon challenge (Section IV, Figs. 5 to 7). Similar results were reported by Wigzell (1966), where antibody was transferred three to six days after the antigen. He found that, when the animals were challenged with antigen at various times after the antibody treatment, expression of memory was nearly completely inhibited,

but recovered with increasing time interval between antibody and challenge. Four weeks after priming, the response of primed animals and that of primed and antibody treated animals to a challenge was similar. These results indicate that either IgG antibodies, given at these times, interfere with the development of memory, or that the antibodies remaining at the time of challenge interact with the antigen and inhibit the secondary response. The recovery of secondary immune potential in Wigzell's (1966) experiments a considerable time after the antibody injection, could mean that memory was present at the earlier times too but its expression was blocked. It could, however, also mean that as the antibodies decayed with time, some antigen remaining in the animals primed more and more virgin cells, resulting in an increased immune potential. Cunningham (1970) has shown, and it has been confirmed in this study, that, at a time when primed animals would not give a secondary response on challenge (Section IV, Fig. 2), cells from these animals transferred to irradiated recipients would

give a good secondary response on challenge (Section IV, Fig. 16). We have further shown that cells from animals which received passive IgG or $F(ab')_2$ antibody, a day after priming, gave as good a secondary IgM response in irradiated recipients as the cells from donors which received antigen. It clearly indicates that passive antibody treatment a day after the priming antigen does not interfere with the development of IgM memory. Similar findings have been reported by Sinclair and Chan (1971). They have shown that transfer of cells from animals primed with SRBC and given passive IgG antibody one day after the antigen did not have any effect on IgM memory, although it did inhibit the development of IgG memory. It may well be that the requirement for antigen for the development of IgM memory is fulfilled within a short time after antigen injection. Under these circumstances, twenty-four hours later, no more antigen may be required for this purpose, and therefore antibody may not have any effect. It is also possible that the antigen re-

quired for priming rapidly localizes in compartments, which are not accessible to antibody, and this localization takes less than twenty-four hours. Another explanation is put forward by Sinclair and Chan (1971). According to these authors, the steps involved in the establishment of IgM memory are not inhibitable by IgG, while those involved in that of IgG memory are inhibited. Whatever the reason for this difference in the effects of IgG, it is highly unlikely that antigen is not involved in these steps.

Nossal and his colleagues (1965) and Wigzell (1966) observed a short life for IgM memory in their systems. Nossal et al. (1964a) in their study of antibody-forming cells had also observed that some IgM forming cells continued to produce the same class of antibodies. Other cells produced IgM for some time and then switched over and started to form IgG antibodies. They believed that the short life of IgM memory is due to this switch-over (Nossal et al., 1965). It is difficult to believe that this is the cause of short IgM memory or absence of IgM memory when large doses of antigen are used for priming. As has already been mentioned, the short life of IgM memory in fact is artifactual, because one can detect

memory cell response if proper environment is provided to them. The second evidence against switch-over hypothesis is the one provided by the limiting dilution assay of Shearer and Cudcowicz (1969). In trying to find out whether direct PFC, indirect PFC, and cluster-forming cells (CFC) arise from the same precursors, these authors have shown that precursors of these cells in normal animals occur at frequencies different from each other. By using a limiting dilution assay, these authors could obtain a direct PFC response alone. The authors have used statistical methods to show that all three of these cells arise from different precursors, which occur independently of each other. Similarly, Eidinger (1968), using lymph node imprints and Jerne plaque technique in a study correlating the morphological and immunological changes various times after primary and secondary antigenic challenge, have obtained evidence that 19S and 7S antibody-forming cells arise from different precursors. Valentova et al. (1966, 1967) observed a short-lived IgM memory in their system. They explained this on the basis of the short life of the IgM memory cell. It is possible that some memory cells have a short life span, probably the ones which arise early in priming. The lack of memory in the intact animals at

later intervals, however, is certainly not due to the absence of memory cells.

Another important aspect of the immune response which has come out from our dose response studies is the reduced responsiveness of the animals upon using a large dose for priming. Similar findings have already been reported by many laboratories (Byers and Sercarz, 1968; Hanna et al., 1969; Hanna and Peters, 1971). It has already been mentioned that the use of a large dose of antigen for priming may result in the production of IgG antibodies, which in turn could inhibit an IgM response (Uhr and Finkelstein, 1967; Uhr, 1968; Wigzell, 1967; Moller and Wigzell, 1965; Rowley and Fitch, 1966). It has been suggested that the use of a large dose of antigen results in the exhaustion of memory cell population, by forcing this cell population to differentiate into antibody-forming cells (Sercarz and Coons, 1962; Byers and Sercarz, 1968; Hanna et al., 1969; Hanna and Peters, 1970).

According to the X-Y-Z scheme of immunocyte maturation postulated by Sercarz and Coons (1962), antigen stimulates the differentiation of the virgin, or X-cells, to the memory or Y-cells. Upon further antigenic stimulation, the Y-cells differentiate into the antibody-forming cells

or Z-cells. A dose of antigen, which is in excess to convert most of the X-cells to Y-cells, would therefore stimulate the differentiation of Y-cells to Z-cells. In such a condition, the Y-cell compartment would be depleted. The degree of depletion would depend upon the amount of antigen given for priming. Since a smaller number of memory cells would be available to respond to the challenge, the use of a large dose for priming would result in a smaller secondary response. According to this scheme, therefore, the transfer of cells from donors primed with a large dose of antigen would also result in a small response. Similar results were obtained when cells from donors primed with a large dose of antigen were transferred into irradiated recipients. It has been shown that treatment of such donors with specific IgG antibody, one day after the antigen, results in an increased response in the cell recipients (Hanna et al., 1969). This observation has been taken to mean that antibody injected one day after the antigen binds excess antigen, which may have stimulated the exhaustive differentiation of memory cells to antibody-forming cells. As a result, antigen is no longer available to deplete the memory cell compartment. Since antibody was injected one day after the antigen sufficient time was

allowed to stimulate the formation of memory cells from the antigen-sensitive X-cells. Similar results were obtained in this study (Section IV, Figs. 21 and 22). However, it would be too simple to interpret these results in such a way, particularly in the light of recent evidence that antigen-sensitive and antibody-forming cells arise from different precursors (Miller and Mitchell, 1968). It is not definitely known whether or not the antibody-forming cells are direct descendents of memory cells. In this regard, too, there is evidence that memory cells belong to thymus-derived cells (Miller, 1971; Moller, 1971). Accordingly, it is very difficult to accept exhaustion of immune response upon large dose priming as accounting for the expenditure of memory cells. Furthermore, we have shown that, although the use of IgG in the donor resulted in the rescue of IgM secondary response in the recipients, the use of $F(ab')_2$ did not rescue the secondary response. Since it is known that IgG antibody inhibits IgG response as well as IgG memory while its $F(ab')_2$ fragment is very poor in this respect (Sinclair, 1969; Sinclair et al., 1970), the rescue of memory by IgG antibody may be a result of suppression of IgG antibody-forming cells. Alternatively, IgG may inhibit the differentiation of Y-cells to Z-cells

upon antigenic stimulation, and this may prevent the depletion of memory cell compartment.

When IgG antibody is injected to the donors one day after priming with a small or medium dose of antigen, the response of the cell recipients is unchanged (Section IV, Figs. 17 and 21). However, when IgG antibody is given to the donors primed with a large dose of antigen, the response of the cell recipients is significantly increased (Section IV, Figs. 21 and 22). On the surface, these two observations seem to contradict each other. However, it becomes apparent that there are important differences in the conditions, which may very well be responsible for the different results. In the first instance, where we have shown that antibody treatment of the donors, one day after the antigen, does not affect the secondary immune response in the cell recipients, the antigen dose gives rise to a small IgG antibody response. Therefore injection of IgG antibody to the cell donors would not have any effect on the response of the recipients, unless it affected IgM priming. It has been shown that injection of IgG to the cell donors does not affect IgM priming (Section IV, Fig. 17 and Sinclair and Chan, 1971). In the second case, where injec-

tions of IgG antibody to the donors rescued the IgM secondary response in the recipients (Section IV, Figs. 21 and 22 and Hanna et al., 1969), an antigen dose which gives substantial IgG response was used for priming. Therefore, in this case, one may have a large number of IgG antibody-forming cells in the cells transferred to the irradiated recipients. These cells could produce IgG antibodies in the recipients, which may inhibit the IgM PFC response. Therefore the cells from donors primed with a large dose of antigen would give a suppressed IgM PFC response. It would therefore seem as if the large dose primed donors do not have memory cells, although it would essentially be an inhibition of expression of memory in the cell recipients. It has been shown that small amounts of IgG antibodies given with challenge antigen result in the inhibition of primary as well as secondary response. Therefore, a small number of IgG antibody-forming cells in the inoculum may prove sufficient to inhibit the response of the cell recipients. The failure of $F(ab')_2$ antibody to rescue memory in the recipients indicates that something more than antigen-binding alone, is responsible for the rescue. It may be argued that the suppressed responsiveness of the cells from animals primed

with a large dose is due to a small amount of IgG antibody carried over in the cell inoculum. It has been shown by washing the cells a number of times before transfer that this may not be the reason for the suppressed response (Section IV, Table I). If carry-over antibody was responsible for the inhibition of response in the recipients, one would expect that the greater number of times the cells were washed before transfer, the more antibody would be washed off, and consequently the recipients would give a larger response. We have failed to observe this relationship between the number of washings and the response of the cell recipients. This indicates that either the antibody carried over in the inoculum is in a form which is not easily washed off the cells, or carry-over antibody is not the cause of inhibition.

It is possible that antigen-antibody complexes, firmly bound to the cells, may be transferred in the cell inoculum. These complexes then could inhibit the immune response (Diener and Feldmann, 1970). It has been shown that antigen associated with the cells from primed donors can be unmasked from the cells by incubating these cells at $37^{o}C$ (Mitchison, 1969). A similar treatment given to cells from animals primed with a large dose did not result

in any increase in the response in recipients.

Further support for IgG antibody-forming cells, or IgG antibody which cannot be washed from the cells, being responsible for the inhibition comes from the cell mixing experiment. It was argued that if tightly bound IgG antibody or IgG antibody-forming cellsin the inoculum is responsible for the decreased response of cells from donors primed with a large dose of antigen, then these cells should be able to inhibit the response of other responsive cells. It was found that, when the cells from animals primed with a large dose of antigen were mixed with cells from animals primed with a medium dose, the response was less than that given by an equal number of cells from donors primed with the medium dose. It was found that this inhibition of response could be achieved even if the cells from the donors primed with the large dose were given 1500 rads of γ-irradiation before mixing with the cells from animals primed with the medium dose. This indicates that multiplication of such Ig G antibody-producing or IgG antibody-carrying cells is not required for inhibition. Therefore only a small number of such cells is sufficient to block the secondary response in the recipients.

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