THE EFFECT OF CONTINUAL ANTIGENIC STIMULATION ON THE IMMUNE SYSTEM OF MICE

by

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ABSTRACT

The effect of continual antigenic stimulation on the immune system of mice was studied using two different experimental approaches. A GVHR was induced in F_1 mice by the injection of parental spleen cells at weekly intervals. Several weeks later the spleen cells of mice undergoing a GVHR were shown to be immunosuppressed as their *in vitro* responses to the mitogens Con A and LPS were substancially lower than control animals. The serum from these treated mice was also immunosuppressive to normal spleen cells. The proliferative response to Con A and allogeneic cells of normal: syngeneic, allogeneic, and parental spleen cells was 90% suppressed when GVH serum was added in comparison to the addition of normal serum. Similarly, the *in vitro* antibody response to a T dependant antigen was impaired; however, the antibody response to a T independant antigen was not impaired. These results indicate that T cell functions are more sensitive than are B cell function to immunosuppressive factors in the serum of mice undergoing a GVHR.

The serum was fractionated by gel filtration on a Bio-Gel P-200 column. The inhibitory material in GVH serum eluted in the immunoglobulin fraction of serum which indicate that it has a molecular weight of 150,000 or greater.

The second approach studied involved continual allogeneic stimulation. Parental type mice were injected at five day intervals with F_1 spleen cells in order to induce a HVG reaction. After several injections the spleen cells from these mice were tested *in vitro*. The spleen cells from HVG mice responded the same as normal spleen cells to the mitogens Con A and LPS. The spleen cells from HVG mice showed an enhanced *in vitro* antibody response as compared to normal spleen cells. This enhancement was attributed to the allogeneic effect.

This series of experiments have shown that the induction of a GVHR in mice can later lead to immunosuppression and production of immunosuppressive

factors in the serum of these mice. The induction of a short term HVG reaction has no adverse effects on the immune system except for enhancing an antibody response. It is possible that a more prolonged HVG reaction would parallel the immunosuppression observed in mice undergoing a GVHR.

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ABBREVIATIONS

Abbreviations used in this thesis GVHD, graft-vs-host disease; GVHR, graft-vs-host reaction; GVH mice; mice undergoing a GVHR, GVH serum, serum from GVH mice; HCG, host-vs-graft; HVG mice, mice undergoing a HVG reaction, PHA, phytohemagglutinin, PBS, phosphate-buffered saline; FCS, fetal calf serum; ConA, concanavalin A; LPS, bacterial lipopolysaccharide; PFC, plaque forming cells; DNP-LPS, dinitrophenylated LPS, SRBC, sheep red blood cells; MLC, mixed lymphocyte culture; ^{3}H-TdR, tritiated thymidine.

INTRODUCTION

The intensity and duration of an immune response is controlled by several regulatory mechanisms. An immune response is normally initiated when immunocompetent cells are confronted with an antigen. Once the antigen is removed the immune response usually subsides, implying that continual antigenic stimulation is necessary for a continual immune response. Control of this level is therefore maintained by the source of antigen available to the lymphoid cells.

Suppression of an antibody response by passive immunization has been observed for many antigens (Rowely, 1973). Relatively small amounts of specific antibody if given within twenty-four hours after immunization will specifically suppress the antibody response to that antigen (Rowely, 1973). This mechanism has been called antibody feedback inhibition and has also been shown to operate in vivo under normal conditions (Graf, 1967).

There is also evidence of a cellular control system. Macrophages can modulate the immune response by processing and presenting antigen to other lymphoid cells. Certain thymus-dependant lymphocytes (suppressor T cells) can directly effect the production of antibody by thymus independant cells (B cells) (Katz, 1972). Under normal physiological conditions an immune system is not confronted with a chronic source of antigen and therefore all the control mechanisms act to regulate an effective immune response.

One model system in which immunocompetent cells are confronted with a continual source of antigen is the graft verses host reaction (GVHR). A GVHR is induced by injection of lymphoid cells, which are capable of engaging in an immune response, into a host which possess antigens distinct from the injected cells. The host must be incapable of mounting an immune response against the grafted cells in order for the grafted cells to survive. The simplest system to study is to inject parental lymphoid cells into a

genetically tolerant F_1 hybrid host.

The term GVHR refers to the immunological response of the donor lymphoid cells to the foreign histocompatibility antigens of the host. The term graft versus host disease (GVHD) refers to the complex syndrome which results in the host as a result of the GVHR (Elkins, 1971).

The Cells which Initiate a GVHR

It has been shown in a number of experiments that T lymphocytes are the cells responsible for a GVHR. Treatment of normal spleen cells with anti-theta serum and complement, which kills the T cell population, will render the cells incapable of initiating a GVHR (Lonai, 1973). Similarly lymphoid cells obtained from adult mice that have undergone neonatal thymecotomy are also incapable of inducing a GVHR (Miller, 1967). Bone marrow cells do not directly induce GVHR. However they contain precursor T cells which are matured by the host's thymus and then are capable of inducing a GVHR (Thomas, 1969). The theory that bone marrow cells require the host's thymus in order to induce a GVHR is supported by the fact that bone marrow cells do not induce a GVHR in thymectomized hosts (Taylor, 1963). More recently it has been shown that there are two types of T cells which might be required to elicit a GVHR. Synergistic responses were obtained when small numbers of cells from lymphoid tissues that were rich in GVH activity, such as spleen and lymph nodes, were combined with a larger number of weekly reactive thymus cells. The degree of synergy was dependant upon the ratio of the two cell types (Cantor, 1970).

Fate of the Host

The induction of a GVHR in a host animal leads to a complex syndrome. Depending on the genetic differences between the host and the donor, and the number of donor cells injected, various lesions occur. With strong genetic differences the GVHD is characterized by loss of body weight, splemomegaly,

hematomegaly, lymph node atrophy, immunosuppression, glomerulonephritis, and eventually death. Depending on the strains of inbred mice used, various syndromes occur. In some strain combination, one injection of donor cells will lead to death within thirty days whereas with other strain combinations after four equivalent injections, the animals remain healthy up to one year after the last injection. Splenomegaly and immunosuppression are early and constant findings with almost every animal undergoing a GVHR. Initially it was assumed that the spleen enlargement resulted from the grafted cells proliferating in response to the host alloantigens (Simonsen, 1957). Investigations using chromosome markers soon indicated that most of the proliferating cells were of host origin (Elkins, 1966). More recently it was shown that grafted cells pretreated with mitomycin-C, which inhibits DNA synthesis, can still induce splenomegaly in F_1 hosts provided they were mixed with F_1 cells overnight prior to injection into host animals (Scollay, 1974). These authors' explanation for their results was that the injected cells can mount an immune response to the host's alloantigens (as far as possible without cell division) and release soluble factors which non specifically induce host cell proliferation. Regardless of whether their explanation is true, splenomagaly appears to be the result of host cell proliferation.

There has been one report in which the host appeared tomount a response to the grafted cells. When parental lymphoid cells were injected into an F_1 host these cells produced antibody against the host (if they were B cells) or become cytotoxic (if they were T cells). The parental cells now have something which the F_1 cells lack themselves. This is the idiotype of the anti-parental antibody and the corresponding receptors on the parent anti-F_1 T and B lymphocytes. It was shown that a few F_1 animals injected with parental cells produced an antibody to this idiotype (Binz, 1975). At this time it remains unclear what role in a GVHR this host response may have.

The Effect of GVHR Upon the Immune Responsiveness of the Host

The immune system of animals undergoing a GVHR has been shown to be severely immunosuppressed. B cell responses, as measured by serum antibody levels and by PFC, to both T dependant antigens (Davies, 1970) and T independant antigen (Moller, 1971) have been shown to be suppressed. Allograft rejection has been found to be prolonged in animals with chronic GVH, implying that T cell function was suppressed under these conditions (Lapp, 1969). In an attempt to overcome the immunosuppression in GVH mice, normal syngeneic lymphoid cells were adoptively transferred into the host mice (Claman, 1969). This transfer did not restore immunocompetence of the host implying that the donated competent cells had become immunosuppressed. In another system it was shown that F_1 animals which had received one injection of parental cells were resistant to a second injection of parental cells which would normally cause acute GVH in untreated animals (Fidd, 1966). Resistance to the second challenge was transferred to normal F_1 animals by parabiosis or cross circulation (Field, 1967). This experiment indicated that the resistant state was due to either inhibitory factors or suppressor cells in the blood of these animals. There have been a few attempts to demonstrate *in vivo* that serum from GVH mice contains factors which can suppress the immune system of normal animals (Grushka, 1974). These experiments were unsuccessful, perhaps due to insufficient amounts of serum transferred. More recently, it was demonstrated that spleen cells from GVH mice when cultured *in vitro* produce a factor(s) which suppresses the response of normal spleen cells to PHA (Phillips, 1975).

In addition to the demonstration of inhibitory factors other workers have recently shown that certain cell populations from the spleens of animals undergoing GVHR can suppress an *in vitro* immune response. It was shown that the adherent cells from these animals, when cultured with normal spleen cells

would suppress the generation of PFC to the T dependant antigen, SRBC (Elie, 1975). The adherent cells from the spleens of animals undergoing a GVHR did not appear to be solely suppressor cells but served to function as regulatory cells in the inductive phase of an immune response. This was based on their results which showed that these adherent cells could replace normal adherent cells if added in certain ratios. They postulated from their in vitro results that an excess number of the adherent cells in the host's spleen may be responsible for the observed in vivo immunosuppression of humoral responses.

Summarizing these findings it appears that the immune system of an animal undergoing a GVHR may be immunosuppressed due to an excess number of suppressor cells which in turn produce an inhibitory factor(s) which suppresses the function of other lymphoid cell populations.

Immunosuppression in Tumour Bearing Animals

Another situation in which animals are subjected to excess antigen are those animals which have a progressively growing tumour. It has been shown that immunosuppression often accompanies tumour growth. Tumour specific immunity which is present during early growth of the tumour has been shown to decrease as the tumour size increases (Deckers, 1973). In some tumour systems, along with the decrease in specific immunity there is also a decrease in general immunocompetence. This general immunocompetence has been measured by lack of ability of spleen cells from tumour bearing mice to respond non specifically to mitogens (Whitney, 1974), to mount a response to allogeneic cells (Haran-Gheru, 1973), and to produce antibody to a defined antigen (Smith, 1973).

There are several possible causes of the immunosuppression which accompanies tumour growth. Previous work has shown that serum from mice with large tumours is capable of suppressing the proliferative response of normal mouse spleen cells to mitogens and allogeneic cells (Whitney, 1975). It has

been found that the material responsible for this inhibition eluted with immunoglobulin on Shephadex G-150. Furthermore, the inhibitory factor could be removed on an anti-mouse immunoglobulin immunoadsorbent (Levy, 1975). These results indicate that the inhibitory substance is antibody - like in nature and could be an antibody-antigen complex or a regulatory antibody.

The spleens, from those animals whose serum is immunosuppressive, contain cells which can suppress the response of normal spleen cells to mitogens (Pope, 1975). The relationship between the suppressor cells and the serum factor is unclear; however one might speculate that the serum factor was a product of the suppressor cells in response to excess antigen. Another source of the serum factor which cannot be ignored is that it could be a product from the tumour cells. This is unlikely, since these suppressive substances can be removed on immunoadsorbent columns prepared with anti-mouse immunoglobulin.

Objective of the Thesis

The objective of this thesis was to study the effects on the immune system of continual antigenic stimulations. Two model systems were used:

(1) A GVHR was induced in F_1 animals by the injection of parental spleen cells. This enabled the grafted cells to be in constant contact with allogeneic antigen while the host was genetically tolerant to the grafted cells.

(2) Animals (mice) were subjected to continual allogeneic stimulation by repeated administration of semi-allogeneic (F_1) spleen cells. This initiated a one way "Host vs. Graft" response since the injected cells were tolerant to the host.

The lymphoid cells of these animals were then assayed by various *in vitro* measurements of immunocompetance. It will be shown that continual allogeneic stimulations has no adverse effects on the immune system of mice while the

induction of a GVHR in mice leads to immunosuppression.

MATERIALS AND METHODS

Animals

BALB/c, DBA/2J, CBA/J, C57BL/6J, and C57BL/6 X DBA/2J F_1 (B6D2 F_1) Female mice (Jackson Laboratory, Bar Harbour, Maine) aged 2-4 months were used throughout this study.

Induction of GVHD

GVHD was induced in F_1 animals by four weekly intraperitoneal injections of 50 x 10^6 parental (DBA/2J) spleen cells. These mice will be referred to as GVH mice. Control mice consisted of F_1 animals injected as above with 50 x 10^6 F_1 spleen cells. Mice undergoing GVHD induced in this way were clinically well, although spleen size at time of sacrifice was moderately enlarged (2-3 fold increase in weight over normal controls).

Induction of Continual Allogeneic Stimulation

Continual allogeneic stimulation was induced in DBA/2J mice by 7 injections, at 5 day intervals of 20 x 10^6 F_1 spleen cells. These mice will be referred to as HVG mice.

Collection and Preparation of Serum

Blood was collected from the retro-orbital sinus of normal, control, and GVH mice 4 and 6 weeks after the last injection of cells. The blood was allowed to clot for 2 hours at 4^{o}C after which the serum was separated and pooled into the three groups. It was heat inactivated at 56^{o}C for 30 min, absorbed with SRBC for 30 min and dialyzed against RPMI 1640 media for 24 hours. Following dialysis the serum was ultracentrifuged at 100,000 x g for 3 hours, filtered through a millipore 0.22 μ filter and stored at -20^{o}C.

In Vitro Mitogen Stimulations

Spleen cells were prepared in PBS + 5% FCS. The cells were centrifuged at 2000 x g for 5 min, resuspended in PBS + 5% FCS, and the viability determined by trypan blue exclusion. Desired number of cells were centrifuged and resuspended in RPMI 1640 medium and dispensed into microtiter plates (Linbro Chemical Co.). Each well contained 5 x 10^5 viable spleen cells in a final volume of 0.25 ml medium with FCS at a final concentration of 2%, and appropriate concentrations of Con A, LPS, or mouse serum. The cultures were incubated at 37°C in a humidified atmosphere of 95% air - 5% CO_2 for 1-4 days as indicated in each experiment. Eighteen hours before the cells were harvested 1.0 µCi of ^{3}H-Thymidine (sp. act. 5.0 Ci/mmol, New England Nuclear, Montreal, Canada) was added to each well. The cells were harvested onto glass fiber filter paper, dried, and the radioactivity determined. Two way MLC were performed as described above by culturing together 2.5 x 10^5 CBA/J spleen cells and 2.5 x 10^5 BALB/c spleen cells and harvesting after 4 days incubation. Tests were run in triplicate, and the results expressed as the mean value. The standard error in all instances was always $\leq$ 10%.

Preparation of DNP-LPS

LPS, E. coli 055:B5, (Difco Labratories) was dissolved to a concentration of 20 mg/ml in borate-saline (pH 8.5). 2,4-dinitrobenzene-sulfonic acid (Eastman Kodak Co.) and sodium carbonate (Fisher Chemicals) were then each added to a final concentration of 20 mg/ml. The reaction was stirred for 24 hours at room temperature and unreacted reagents were removed by exhaustive dialysis against borate-saline for 48 hours. The DNP-LPS was then sterilized by millipore filtration and stored at -20°C. It has been shown that DNP-LPS cultured with normal spleen cells at sub mitogenic doses invokes a DNP specific antibody response without the help of T lymphocytes (T independant response); (Jacobs, 1975).

In Vitro Antibody Production

Spleen cells were prepared in RPMI 1640 + 5% FCS buffered with 35 mM Hepes buffer. Cells were cultured at a concentration of 10×10^6 in 35 mm petri plates (Falcon #3301) containing 2.0 ml RPMI 1640 media supplemented with 10% FCS (Microbiological Associates #84557), 5×10^{-5} M 2-mercaptoethanol, and varying percentages of mouse serum. Antigens used were either 5×10^6 SRBC or 0.10 µg/ml DNP-LPS. Cultures were incubated at 37°C on a rocking platform for 4 days. The number of direct PFC was determined with a microscope slide assay (Cunninghamm, 1968). DNP plaques were determined using SRBC coated with dinitrophenylated rabbit anti-SRBC-Fab[1] (Strausbach, 1970). Specific anti-DNP plaques were enumerated by subtracting background SRBC plaques from the total number obtained.

Serum Fractionation

Samples of both normal F_1 and GVH serum were chromatographed on an acending Bio-Gel P-200 column (2.6 cm x 90 cm) using borate-saline (pH 8.5) as the eluting buffer. The sample volumes were 1 ml, elution flow rate was 10 ml/hour and 2.0 ml fractions collected. Peaks of 280 nm absorbing material eluting from the column were pooled and concentrated by precipitation with 50% saturated ammonium sulfate. The precipitates were kept at 4°C for 18 hours then centrifuged at 18,000 x g for 20 min. The pellets were dissolved in borate-saline and dialyzed against the same. The dialyzed materials were made up to the original volumes of the serum sample, filtered sterilized, and stored at -20°C.

To assay for inhibitory action, the appropriate fractions were diluted in 1640 medium and added to spleen cell cultures exactly as described above.

RESULTS

Immune Responses of Animals Undergoing GVHD

The spleen cells of control and GVH mice, obtained 8 weeks after the last injection of cells, were stimulated *in vitro* with the T cell mitogen Con A, and the B cell mitogen LPS. Both control and GVH cells follow similar kinetics shown in Fig. 1 as the maximal ^{3}H-Thymidine incorporation occurred on day 3 for each mitogen and each cell type. The maximum Con A response of cells from GVH mice was 40% of the maximum response of cells from control mice. The LPS response of GVH cells was somewhat less severely suppressed, being 73% of the response of control cells. The suppression of GVH cells is not due to a difference in dose response. Cells from control and GVH mice respond optimally to the same concentration of Con A and to the same concentrations of LPS (Fig. 2). Preliminary data showed that these dose responses are the same regardless of the day on which the cells were harvested.

Immune Responses of Animals Undergoing Continual Allogeneic Stimulation (HVG)

The spleen cells of normal and HVG mice, obtained 4 days after the last injection of F_1 cells, were stimulated *in vitro* with Con A and LPS. These results are shown in Table I. Cells from the HVG animals showed a higher response to both Con A and LPS and also had a higher spontaneous ^{3}H-Thymidine uptake.

Spleen cells from normal and HVG mice were also tested for an *in vitro* antibody response to SRBC. These results are also shown in Table I. The cells from HVG mice showed a much higher antibody response, being almost twice as high as the response of normal cells.

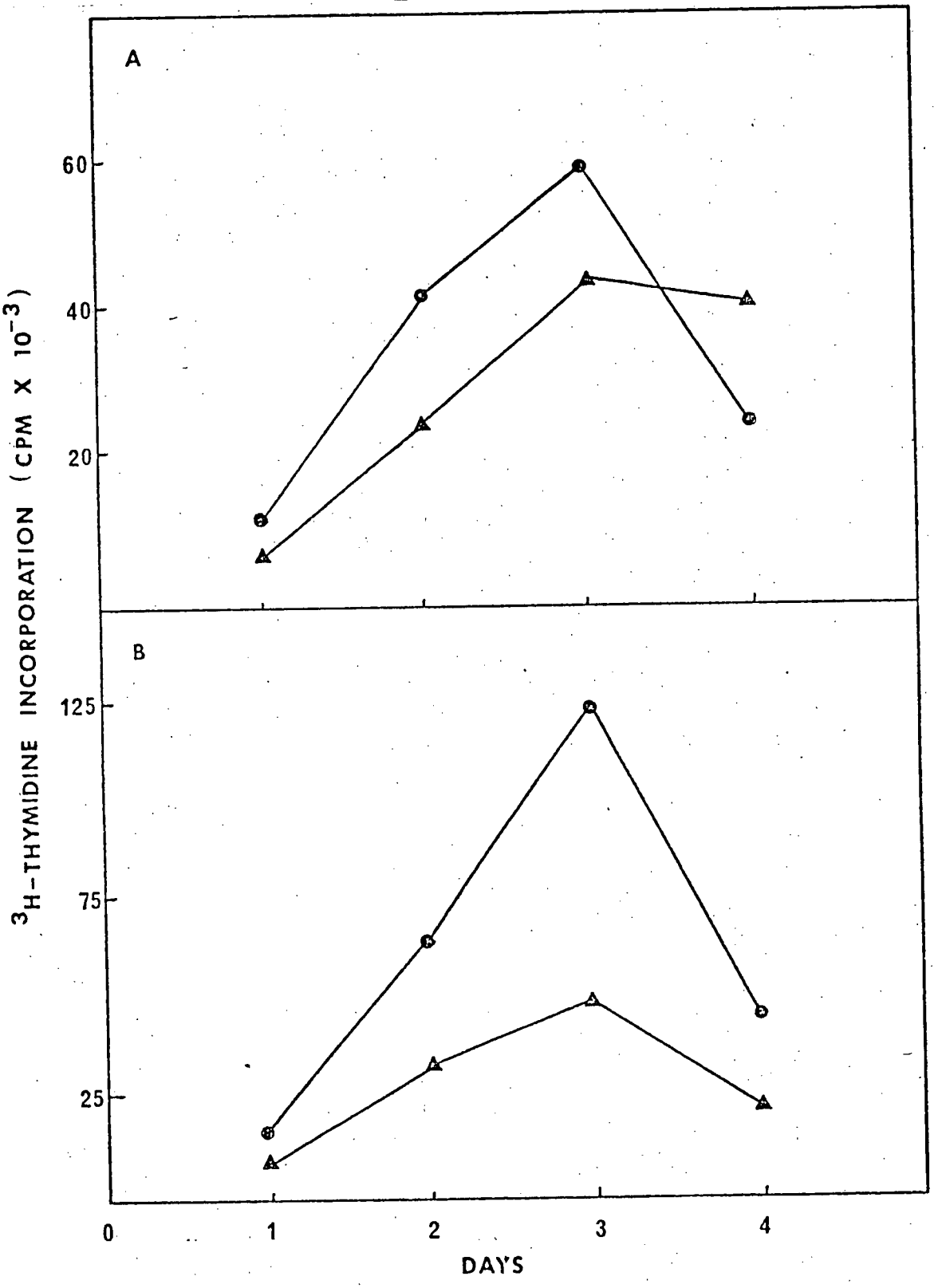

FIGURE 1.

Kinetics of mitogen stimulations of spleen cells from control mice (data from 4 mice), ●---●; and of spleen cells from GVH mice (data from 9 mice), ▲---▲. (A) cells cultured with 20 µg/ml LPS. (B) cells cultured with 2.0 µg/ml Con A.

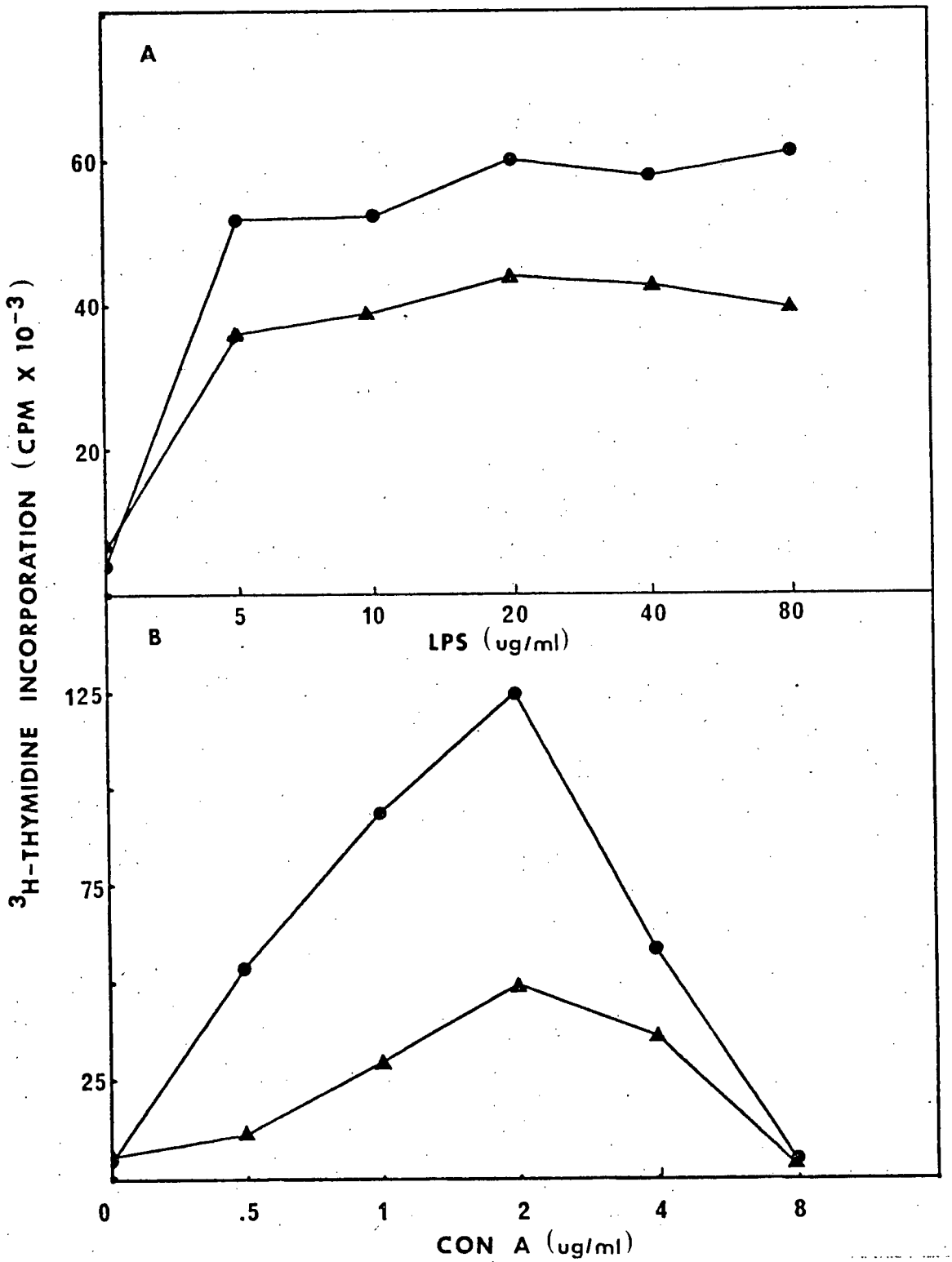

FIGURE 2.

Dose response of mitogen stimulations of spleen cells from control mice (data from 4 mice), ●---●; and of spleen cells from GVH mice (data from 9 mice), ▲---▲. (A) LPS dose response (B) Con A dose response.

IN VITRO IMMUNE RESPONSES OF

SPLEEN CELLS FROM HVG MICE

Spleen Cells From	Mitogen Responses ^{3}H-TdR Uptake (cpm)			Antibody Responses Pfc/culture	
	Medium	LPS	Con A	Medium	SRBC
Normal Mice	8,720	53,177	82,093	226	4,146
HVG Mice	17,789	61,594	125,245	526	8,737

Table I

The in vitro mitogen and antibody responses of spleen cells from HVG mice.

Effects of Serum from Normal and GVH Mice of the Immune Responses of Normal Lymphocytes

The following experiments were designed to test whether GVH serum is immunosuppressive to lymphoid cells from normal mice. Serum obtained from normal, control, or GVH mice was cultured with normal F_1, or DBA/2J spleen cells and various amounts of Con A. The results of these experiments are shown in Fig. 3.

The optimal response to Con A of normal F_1 spleen cells was suppressed by the addition of GVH serum as compared to the additions of normal serum. The response of cells cultured with 2% GVH serum was 30% of the response of cells cultured with 2% normal serum. Increasing the concentration of GVH serum to 4% was even more immunosuppressive as the response was 9% of the response of cells cultured with 4% normal serum. GVH serum also inhibited the Con A response of normal DBA/2J spleen cells which are the same genotype as those cells used to induce the GVH. The optimal Con A response of these cells cultured with 4% GVH serum was 60% of the response of cells cultured with 4% normal serum. In these and other experiments cultures containing normal serum gave the same results as cultures containing control serum, therefore the control in all future experiments is termed normal serum.

Neither of the mouse sera were cytotoxic to normal F_1 spleen cells, as the viabilities of cells cultured with normal or GVH serum paralleled the viabilities of cells cultured alone with FCS (Fig. 4).

GVH serum was also tested for its ability to inhibit the mixed lymphocyte reaction between allogeneic cells. These results are shown in Fig. 5 and are expressed as a percentage response of cultures containing normal mouse serum. As is evident, at 4% GVH serum concentration the response is 93% suppressed.

Further experiments were carried out to determine whether GVH serum inhibited T cell function and/or B cell function, by assaying for the in

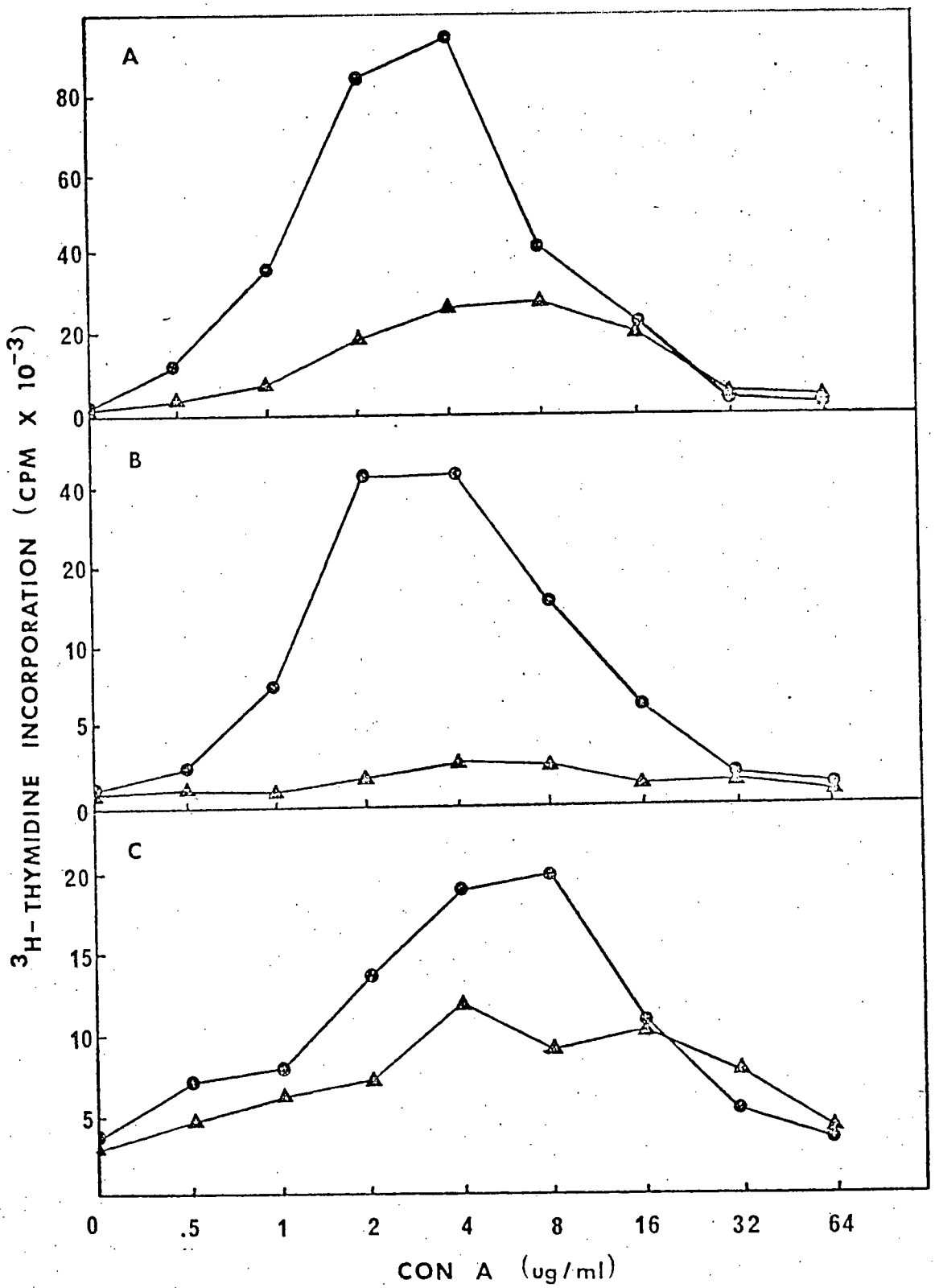

FIGURE 3.

Stimulation of normal spleen cells by various amounts of Con A in the presense of serum from normal mice, ●---●; or in the presence of serum from GVH mice, ▲---▲. (A) normal B6D2 F_1 spleen cells cultured with 2% mouse serum. (B) normal B6D2 F_1 spleen cells cultured with 4% mouse serum. (C) normal DBA/2J spleen cells cultured with 4% mouse serum.

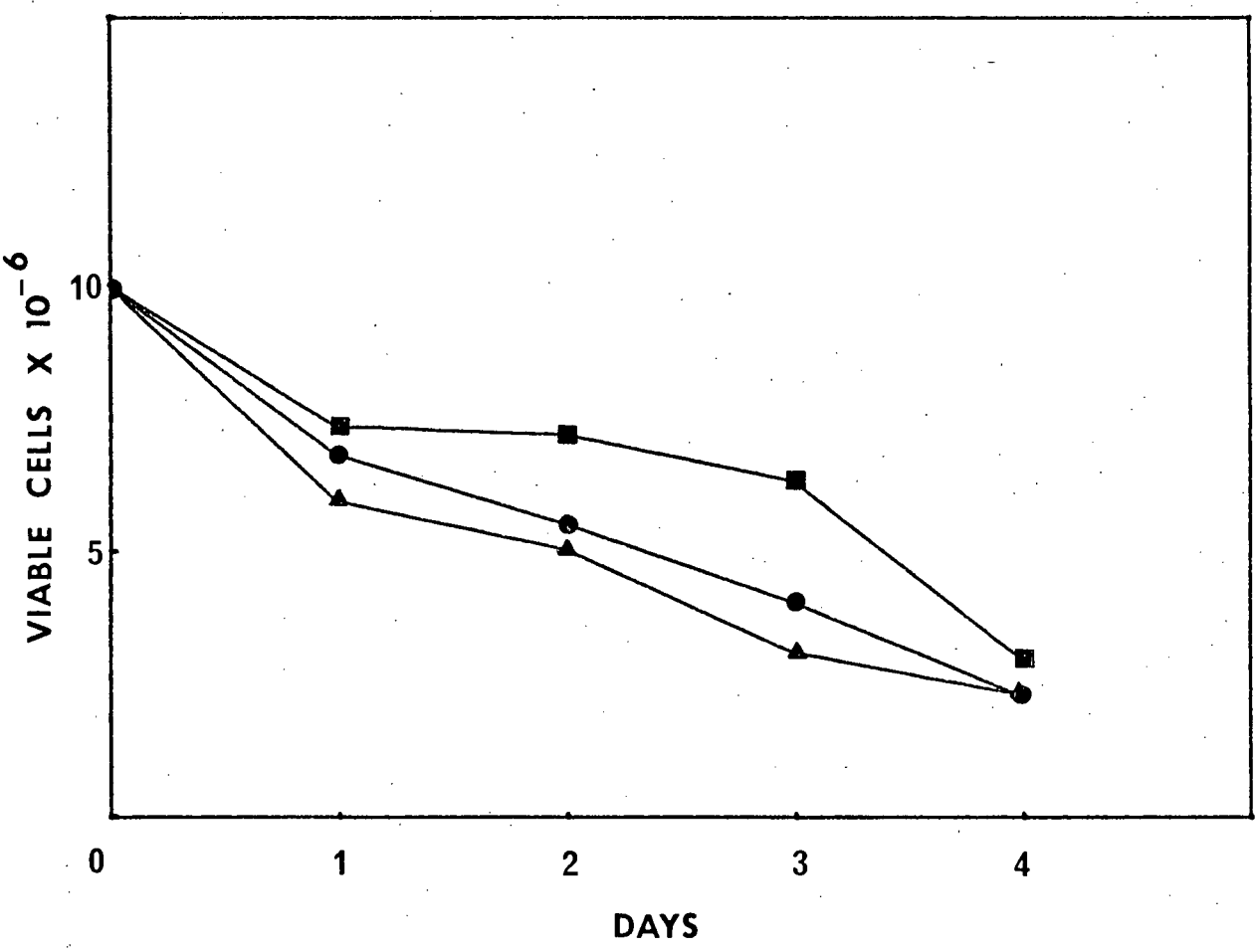

FIGURE 4.

Viability of normal B6D2 F_1 spleen cells cultured with 0% mouse serum, ■---■; cultured with 4% serum from normal mice, ●---●; and cultured with 4% serum from GVH mice, ▲---▲.

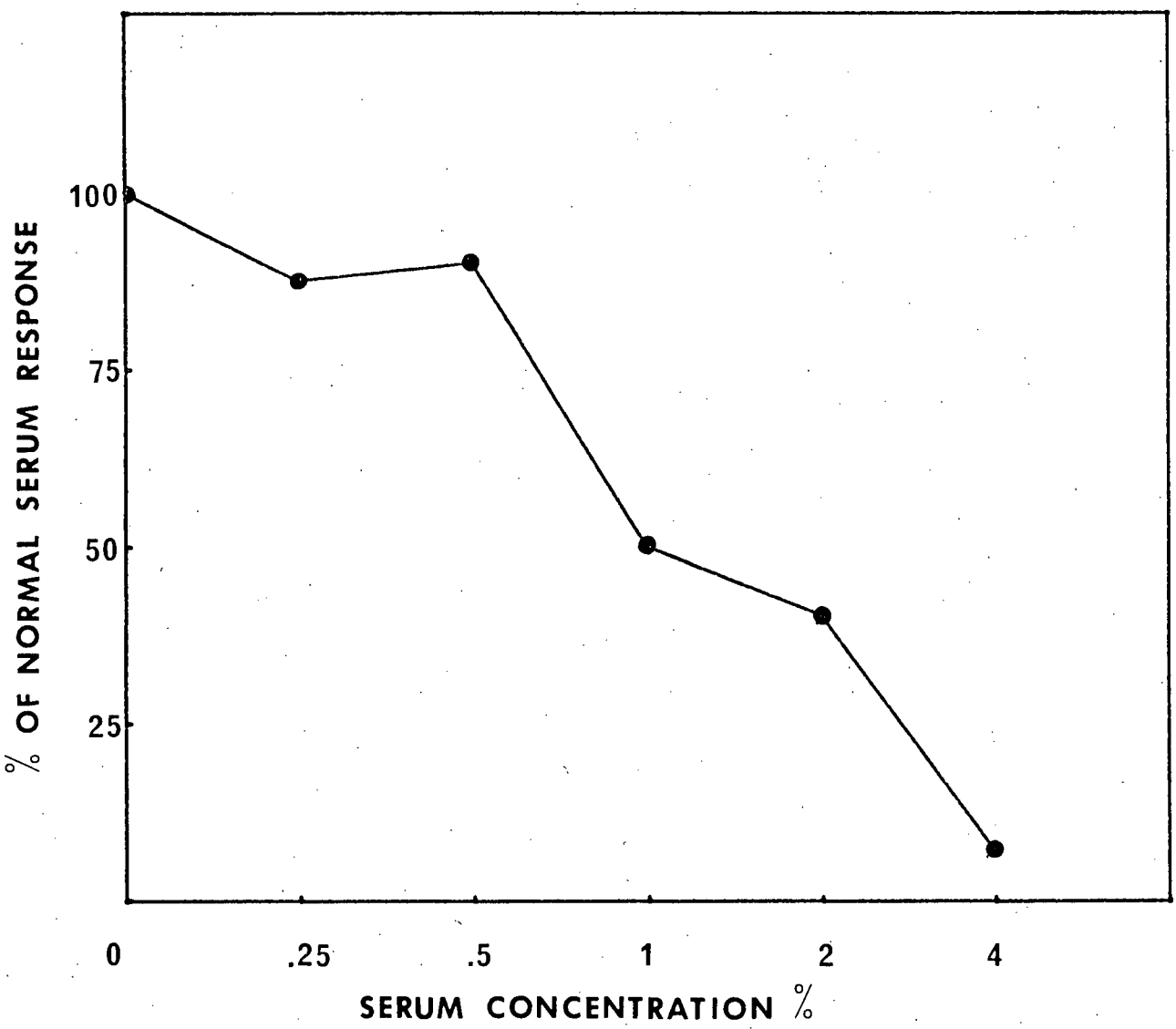

FIGURE 5.

Inhibition by serum from GVH mice of the mixed lymphocyte reaction between Balb/c and BCA/J spleen cells. The results are expressed as % of the response of spleen cells cultured with corresponding amounts of serum from normal mice.

vitro generation of PFC in its presence. Spleen cells from normal F_1 mice were cultured with various percentages of normal or GVH serum and stimulated with either the T dependent antigen, SRBC, or the T independent antigen, DNP-LPS. These results are shown in Table 2. Although both normal and GVH serum inhibit the response to SRBC, at equal concentrations the GVH serum is always much more suppressive. The response to DNP-LPS was not suppressed by any concentration of GVH serum assayed but showed a somewhat depressed response with 4% normal serum.

Gel Filtration of Serum

Figure 6 shows the elution profile of normal and GVH serum eluted from a Bio-Gel P-200 column. The first peak corresponds to the immunoglobulin fraction of serum which contains molecules having a molecular weight of approximately 150,000 or greater. The second peak corresponds to the albumin fraction of serum which contains molecules having a molecular weight smaller than 100,000. The first peak of GVH serum appears to have more material than does peak 1 of normal serum while the second peaks appear to equal amounts of material.

Assay of Fractionated Serum

The P-200 fractions of normal and GVH serum were tested for their ability to inhibit the proliferative response of normal F_1 spleen cells to Con A. These results are shown in Figure 7. At higher concentrations, fraction I from GVH serum, was immunosuppressive, inhibiting the Con A response by 40% as compared to fraction I from normal serum. By expressing the results as a percent of response obtained with normal fractions it appears that fraction II from GVH serum is stimulatory. However the total uptake of ^{3}H-thymidine was always higher in control cultures which did not contain any mouse serum fractions. Fraction II from GVH serum is therefore not as suppressive as fraction II from normal serum.

EFFECT OF SERUM FROM NORMAL AND GVH MICE

ON THE IN VITRO PRIMARY PFC RESPONSE

% Serum Added	SRBC PFC/CULTURE		DNP PFC/CULTURE	
	Normal	GVH	Normal	GVH
0.5	4350 ± 665	2750 ± 269	5544 ± 1053	5048 ± 155
1.0	4166 ± 604	1278 ± 111	5925 ± 420	6743 ± 283
2.0	2641 ± 634	471 ± 40	6049 ± 241	6898 ± 641
4.0	695 ± 69	76 ± 11	3717 ± 1115	6357 ± 340

Table II

The influence of serum from normal and GVH mice on the development of PFC in vitro to SRBC and DNP-LPS. The response in unstimulated cultures was always < 10% of the corresponding stimulated culture.

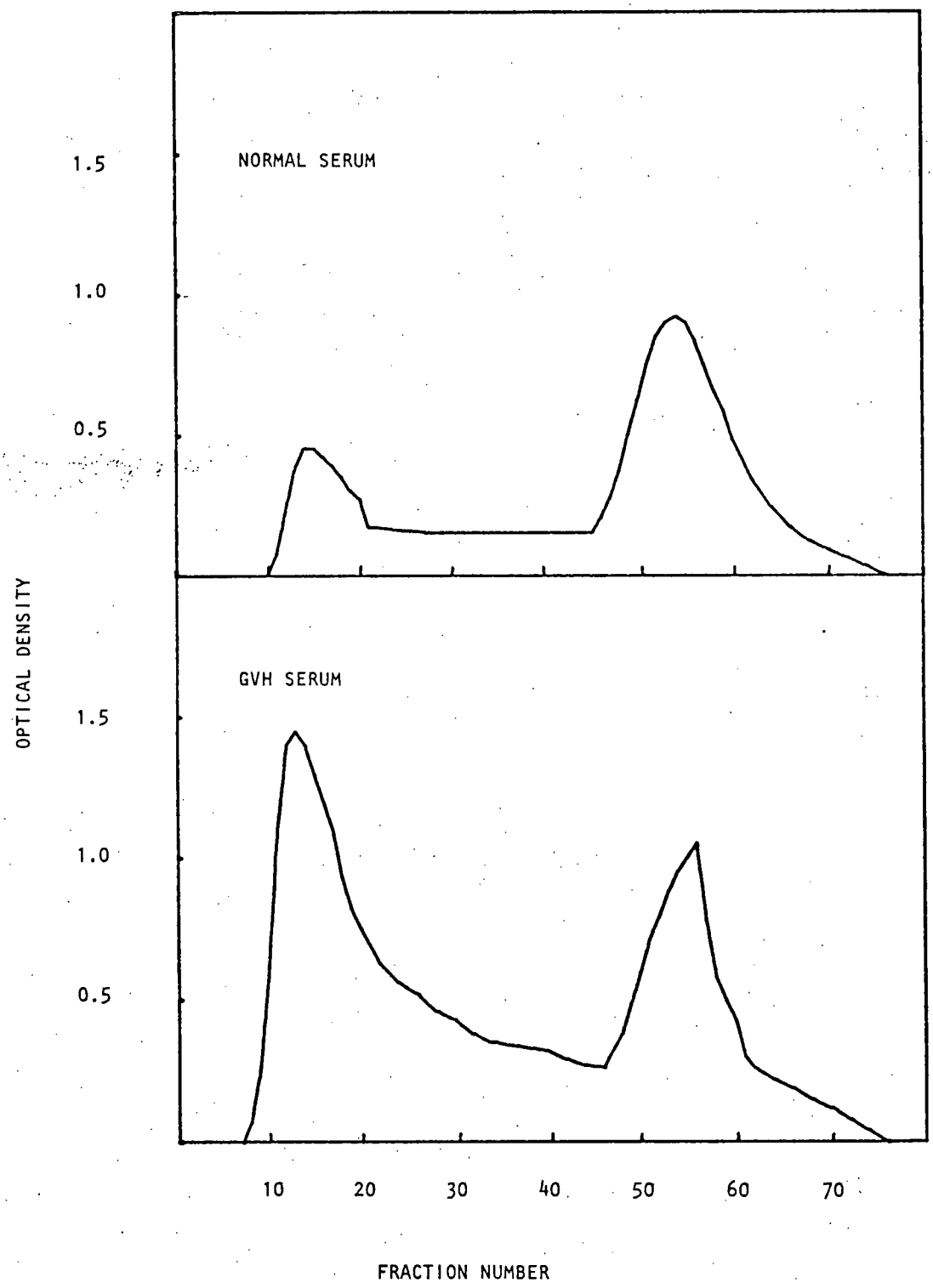

FIGURE 6.

Elution profile of normal and GVH serum from a Bio-Gel P-200 column.

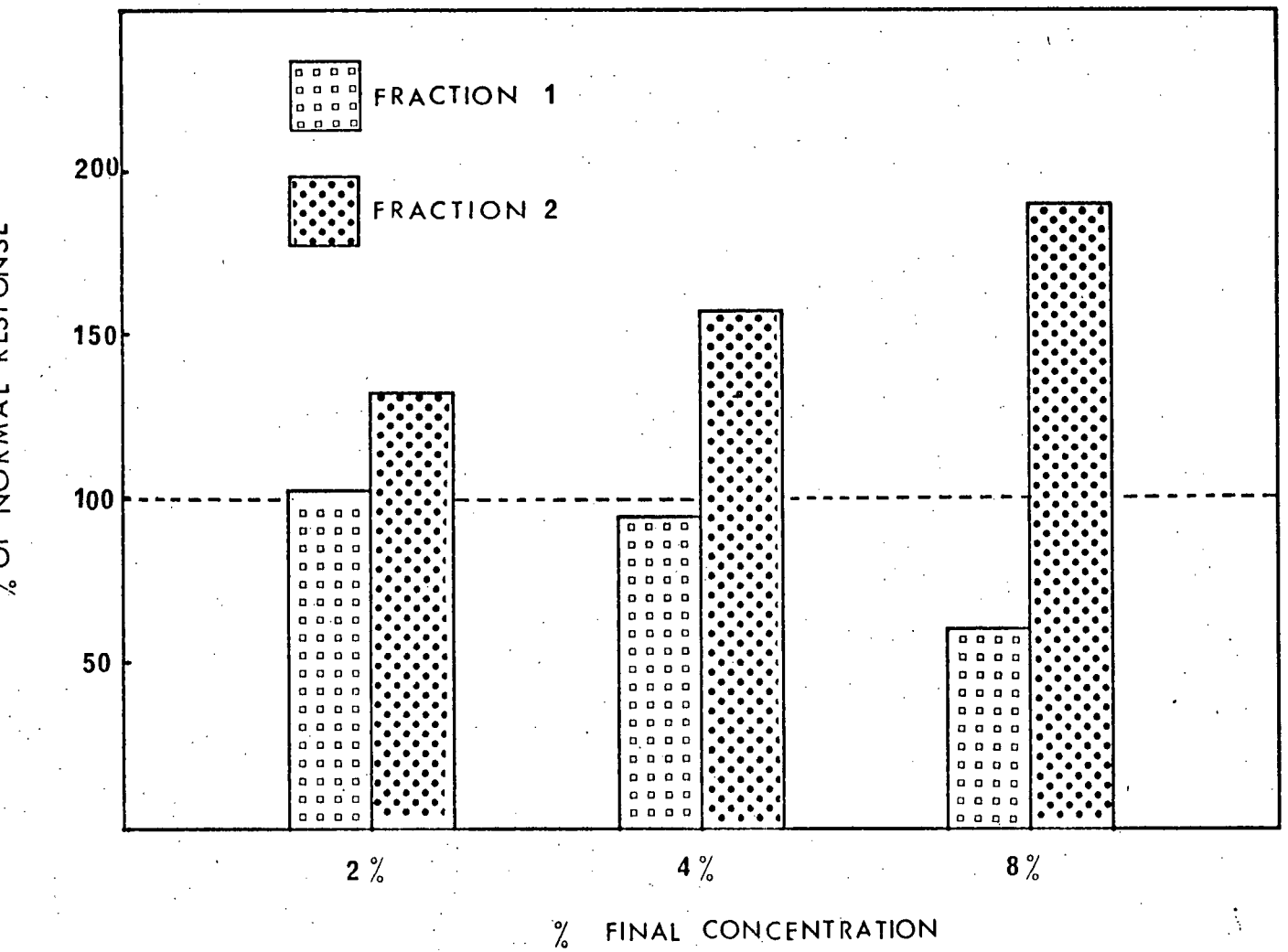

FIGURE 7.

Effect of fraction I and II from Bio-Gel P-200 on the Con A response of normal F_1 spleen cells. The results are expressed as % of the response obtained with an equivalent fraction of normal serum in culture with normal lymphocytes.

DISCUSSION

The results shown in Fig. 1 and 2 demonstrate that the spleen cells from GVH mice are impaired in their ability to proliferate *in vitro* in response to mitogens. T cells may be more affected than B cells because the Con A responses are more suppressed than the LPS responses. These results correlate well with numerous *in vivo* studies which show that both T cell (Lapp, 1969) and B cell (Davies, 1970 and Möller, 1971) responses are suppressed in GVH mice.

Recent studies on murine leukemia virus (MuLV), which is activated in some mice by chronic GVH disease, also showed that the spleen cells of these mice are immunosuppressed (Phillips, 1975). It has been reported that in the mouse strain combinations used in the present study, less than 40% of GVH mice had detectable levels of MuLV (Phillips, 1975). It is unlikely that immunosuppression observed in GVH mice is due to MuLV because one would expect, with the strain combinations used in this study, less than 40% of the mice to be immunosuppressed, whereas it was found that every GVH mouse was immunosuppressed.

The spleen cells from mice injected with allogeneic F_1 cells show a normal or increased *in vitro* response to mitogens as compared to untreated mice. The increase in ^{3}H-Thymidine uptake in unstimulated cultures of injected mice is probably the result of an ongoing immune response to the allogeneic cells. The increased mitogen responses may be a reflection that there is a more active population of T and B cells in the spleens of these animals.

The increased number of PFC in cultures from injected mice can be attributed to the "allogeneic effect." This enhancement of *in vitro* antibody responses is the result of non-specific enhancing factors secreted by allo-activated T cells (Katz, 1972).

It becomes apparent that a continual allogeneic stimulation has no deleterious effects on the immune system of such treated mice. A GVHR does

however have some effect on the immune system of these injected mice. In this situation the mice become immunosuppressed, however this may be beneficial in that the grafted cells also become immunosuppressed and therefore are no longer capable of reacting against the host.

The mice undergoing a GVHR were not immunosuppressed until several weeks after the initiation of the GVHR. Perhaps if the allogeneic stimulation was carried out for this same length of time, immunosuppressions would have also occurred.

The serum, from animals whose spleen cells are immunosuppressed due to GVHD, is capable of inhibiting the function of normal syngeneic, semi-syngeneic, and allogeneic spleen cells. The suppressive ability of this serum cannot be directly attributed to infectious virus because the serum was ultra-centrifuged before it was assayed. Similarly, low molecular weight toxic by products not removed by the kidneys which are sometimes damaged by chronic GVH (Lewis, 1968) and steroids would have been removed during the dialysis against tissue culture medium.

At the concentrations assayed, GVH serum appears to affect T cell function rather than B cell function in that it inhibits the proliferative response of normal spleen cells to Con A, inhibits the mixed lymphocyte reaction, and inhibits the antibody response to a T dependent antigen but not to a T independent antigen. At higher serum concentrations B cell function might become suppressed indicating T cells are more sensitive to the serum factor or else there may be another factor affecting B cells. It cannot be ruled out at this time that the inhibitory material could be affecting macrophages which are sometimes necessary for the generation of antibody responses. However, the addition of 2 mercaptoethanol, which was included in the cultures, has been shown to eliminate the need for macrophages in vitro (Chen, 1972).

The inhibitory effects of GVH serum is not H-2 specific because it caused a 93% inhibition of the 2 way MLC in which one of the responding cells

was allogeneic to the source of serum. Only a 50% inhibition of the MLC would have occurred if the serum inhibitor was H-2 specific. The serum factor responsible for the immunosuppression does not appear to be alloantibody, which is sometimes present in the serum of GVH mice (Lapp, W.S., Personal Communication), because the GVH serum inhibited the Con A response of normal DBA/2J cells, which are of the same genotype as the cells used to induce the GVH.

The inhibitory material in the serum of GVH mice eluted on a Bio-Gel P-200 column with the immunoglobulin fraction of serum. This indicates that the inhibitory material has a molecular weight of 150,000 or greater. Although immunoglobulin is the main component of this fraction there are several other proteins with similar molecular weights such as the complement proteins. Further purification of the inhibitory material is needed before one can conclude any additional information on it's chemical nature and source.

It is plausable to assume that inhibitory material in the serum of GVH mice is responsible for the generalized immunosuppression which accompanies GVHD. A similar factor(s), obtained from cell free supernants of cultured spleen cells from GVH mice, has been recently reported which suppresses the PHA response of normal spleen cells (Phillips, 1975). This factor(s) might be an immunoregulatory molecule produced by suppressor cells or it may activate suppressor cells which in turn suppress the function of other lymphoid cells.

It has also been found that the serum of tumour bearing animals also contains a factor which similarly elutes with immunoglobulin and suppresses the function of normal lymphoid cells (Levy, 1975). These immunoregulating molecules may be produced by a host in response to an abnormal antigenic load such as tumour or in the case of an animal undergoing a GVHR be produced by the grafted cells which are in constant contact with foreign antigen.

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