ANTIBODY-MEDIATED SUPPRESSION OF THE IMMUNE RESPONSE IN VITRO

I. Evidence for a Central Effect*

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The immune response may be specifically inhibited by antigen or by antibody. Most immunological phenomena, such as the primary and the secondary antibody response (1, 2), delayed hypersensitivity (3), renal allograft rejection (4), and tumor rejection (5) may be suppressed in vivo by adequate amounts of passive antibody. Such suppression is most readily achieved by antibody administered prior to the antigenic stimulus. The finding that immune suppression can also be obtained by passive antibody given subsequent to the antigen has led to the suggestion that the animal's own antibody may cause cessation of the antigenic stimulus, and thus have a regulatory function (1, 6, 7).

Two theories attempt to explain the mechanism of antibody-mediated suppression. One is that passive antibody competes with immunocompetent cells for antigenic determinants by the formation of antigen-antibody complexes. It is implied that most of these complexes are nonimmunogenic and are degraded by the animal without the release of immunogenic material (8). This theory has been termed "peripheral" in the sense that the immune potential of lymphoid cells capable of reacting to a particular antigen is believed to be undiminished.

Another theory suggests that passive antibody induces a reduction in the number of immunocompetent cells capable of responding to a given antigen (9). Such a mechanism would, at the cellular level, be similar to that of immunological tolerance, which is also thought to be a "central" specific nonreactivity of the immune system (10, 11).

Work on antibody-mediated suppression has to date been in vivo. Due to the complexity of the whole organism, including uncontrollable cell migration and nonhomogeneous distribution of antigen and antibody, the interpretation of in vivo results is difficult. The extrapolation of these results to mechanisms operating at the single cell level has led to contradictory viewpoints. In this paper we

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have investigated the mechanism of antibody-mediated suppression at the cellular level by using in vitro techniques (12–14). The immune responses of mouse spleen cells interacting with antigen and/or antibody for defined periods of time were measured and the parameters of antibody-mediated suppression defined. Use was made of methods for the enumeration of immunocompetent cells and antibody-forming cells to both sheep erythrocytes (15–17) and polymerized flagellin (18, 19). The in vitro studies were extended by transferring cultured cells into lethally irradiated recipients and testing their reactivity in vivo.

Evidence will be presented that antibody-mediated suppression of the immune response acts at the level of the immunocompetent cell in vitro and in vivo. The possible significance of these findings will be discussed in the light of current theories of the mechanism of antibody-mediated suppression and of immunological tolerance.

Materials and Methods

Cell Culture.—The method used was based on that described by Marbrook (13) and modified by Diener and Armstrong (14). A spleen cell suspension was placed in a tube sealed off by a dialysis membrane and suspended from the stopper of an Erlenmeyer type flask containing tissue culture medium. Cultures were placed in a humidified incubator at 37°C, with a gas flow of 10% CO_2, 7% O_2, and 83% N_2.

Tissue Culture Medium.—The medium used was as described by Diener and Armstrong (11), with the exception that it contained 5% fetal calf serum, and in some cases was supplemented with 20 mg of L-asparagine and 100,000 IU of Mycostatin (E. R. Squibb & Sons, Liverpool, England) per liter.

Mice.—CBA mice were used throughout. Tissues for culture were obtained from mice of either sex in the age range 70–110 days. Irradiated recipients were aged 90–180 days. Cell donors and recipients were always of the same sex. Some of the irradiated mice were given Penicillin G in the drinking water (crystalline penicillin G, Evans Medical Australia, Proprietary Ltd., Sydney, Australia).

Cell Suspensions.—Mice were killed by cervical dislocation and the spleens were removed aseptically, minced, and strained through a fine stainless steel sieve into cold tissue culture medium. Cells were counted in a hemocytometer and their viability tested by eosin dye exclusion (20). Usually 15×10^6 spleen cells were placed in 1 ml of medium for each tissue culture.

Antigens.—Purified flagellin (H antigen) was prepared from *Salmonella adelaide* and *Salmonella waycross* by the method of Ada, Nossal, Pye, and Abbott (21). In each case it was used in the polymerized form. Sterile antigen was obtained by filtration of flagellin through a millipore membrane of 0.45 pore size before polymerization. Dilutions were made in sterile double distilled water containing 0.1% fetal calf serum and kept at −20°C. Unless otherwise stated, the immunogenic dose of polymerized flagellin of *S. adelaide* (POL)[1] used in tissue culture was 20 nanogram (ng) per ml.

Sheep erythrocytes (SRC) were collected into Alsever's solution and kept at 4°C for at least

[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; anti-POL, isologous antiserum to POL; anti-SRC, isologous antiserum to SRC; ARC, antigen-reactive cells; μg, microgram (10^{-6}g); ng, nanogram (10^{-9}g); POL, polymerized flagellin of *Salmonella adelaide*; SRC, sheep red cells.

1 wk prior to use. They were washed three times in saline (0.9% NaCl in distilled water) and diluted in medium to a concentration of 4×10^6 cells per culture.

Bacteria.—*Salmonella derby* (H antigen fg; O antigen 1,4,12) served as the indicator strain for detecting antibody-forming cells (AFC) to polymerized flagellin from *S. adelaide* (H antigen fg; O antigen 35).

Assay for the Enumeration of Antibody-forming Cells.—After the appropriate incubation period, cell suspensions were collected separately from each culture, washed twice, and assayed for antibody-forming cells. Antibody-forming cells to POL were assayed by the immunocytoadherence method of Diener (18).

Antibody-forming cells to SRC were enumerated by Cunningham's modification (17) of the hemolysin plaque assay of Jerne and Nordin (22).

Background responses were determined in cultures of cells incubated in the absence of both antigens. The background to POL after 4 days in culture was very low, being less than 0.1 adherence colonies/10^6 cells harvested. The background response to SRC was higher, varying markedly with the batch of fetal calf serum used. In the experiments described in this report, the background (after 4 days of culture) ranged from 0.1 to 6 plaque-forming cells per 10^6 harvested. There was no background detectable at day 1. Background was not subtracted from values in tables or graphs.

Immune Sera.—Male and female CBA mice aged 6–12 months were injected intraperitoneally (i.p.) with 25 μg of POL in Freund's complete adjuvant. They were boosted with 25 μg POL i.p. 3–4 wk later and exsanguinated 1–2 wk thereafter. The sera were separated within 24 hr, pooled, and kept in small portions at −20°C. The titer of two such pools was 10^5 as measured by the immobilization titration method (21). The sera were inactivated by 30 min of incubation at 56°C.

A similar group of mice was immunized with polymerized flagellin from *S. Waycross* (H antigen Z4, Z 23).

Another group of mice was hyperimmunized against sheep erythrocytes by receiving three courses of thrice weekly i.p. injections of 0.1 ml of a suspension containing 20% SRC, with weekly intervals between each course. The mice were exsanguinated 10 days after the last injection. Sera were pooled and kept at −20°C. The hemagglutinin titer of two such pools were 16,000 and 32,000.

For use in tissue culture, immune sera were diluted in culture medium and sterilized by passage through a millipore filter of 0.45 μ pore size. Unless otherwise stated, the concentration in tissue culture of antipolymer antibody was a titer (reciprocal) of 10, and that of anti-sheep cell hemagglutinin one of 8–10.

Iodination of anti-POL.—Anti-POL was fractionated on Sephadex G-200 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). Fractions containing IgG were pooled. A sample was iodinated at a substitution rate of 16 μCi/μg by the chloramine-T method, using carrier-free ^{125}I (Radiochemical Centre, Amersham, Buckinghamshire, England).

Titrations.—Antibody to polymerized flagellin was assayed by the immobilization titration method of Ada, Nossal, Pye, and Abbott (21). The endpoint was a standard degree of immobilization. Hemagglutinins were determined by serial two-fold dilutions of the antiserum in saline, followed by the addition of an equal volume of a 2% solution of SRC. The endpoint was taken as the last tube showing visible agglutination. Titrations were performed using an initial dilution of 1 in 5. For computation of the geometric mean, titers of less than 5 were arbitrarily given the value of 1. Titers were expressed as the reciprocals of the dilution of the titration endpoint.

Enumeration of Immunocompetent Cells Reactive to POL.—This method was recently described in detail by Armstrong and Diener (19). Basically, lymphoid cells were injected intravenously into the lateral tail veins of lethally irradiated syngeneic recipients. 6–24 hr later, 25 μg of POL was injected i.p., and 7 days thereafter the spleen was removed and cut into 260

μ slices by a tissue chopper. The slices were placed in sequence on a slide coated with viable *S. derby* in motility agar (0.6% gelatine and 0.4% shredded agar in heart infusion broth), overlaid with motility agar, and incubated at 37°C for 3 hr to allow bacteria to grow and migrate into the upper layer of agar. The slides were then kept overnight at 20°C so that bacteria could grow into a thick film. Areas of antibody production were characterized by clear translucent areas, denoting a failure of bacterial growth.

A focus was taken as one or more contiguous spleen slices producing antibody, separated from another such area by at least one negative (non–antibody-producing) slice. It has been shown for both antigens, POL and SRC, that there is a linear relationship between the number of lymphoid cells injected into an irradiated recipient and the number of foci produced in the spleen (15, 16, 19). This relationship suggests that each focus is derived from one immunocompetent cell reacting to the antigen.

The adoptive immune response of transferred lymphoid cells was also assessed by measuring the specific activity. The specific activity represents the number of positive (antibody-producing) slices, expressed as a percentage of the total number of spleen slices. The measurement of specific activity was useful, as it permitted the assessment of the immune response of a larger number of injected cells. Claman, Chaperon, and Triplett (23) have found the specific activity of an adoptive immune response to SRC to be linearly related to the number of foci produced.

The ARC background response to POL of mice not injected with any cells was determined in all assays. This was found to be less than 0.05 foci per spleen.

Enumeration of Immunocompetent Cells Reactive to Sheep Erythrocytes.—The method used was a modification of that of Kennedy et al. (15), and Playfair et al. (16), as described by Mitchell and Miller (24). Briefly, spleen slices were placed in sequence on an agar base in a Petri dish, this being covered by a layer of 1.4% agar in Eagle's Basal Medium, containing 2 mg dextran and 0.2 ml of a 20% suspension of SRC. After incubation for 2 hr, 2 ml of guinea pig serum diluted 1:10 was added as a complement source, and the dish incubated for a further hr. To increase contrast, the preparations were stained with benzidine (1 part 5% hydrogen peroxide to 9 parts of 0.55% benzidine in 12.5% glacial acetic acid) for 1 min. The background response to SRC (i.e., of mice not injected with cells) was less than 0.2 foci per spleen. This was not subtracted from the values reported in the tables.

Irradiation.—Mice were placed in groups of 10 in plastic containers and irradiated with a whole body dose of 850 rad. A Philips (RT 250) 250 Kev machine was used and irradiation performed at 15 mA and a half value layer of 0.8 mm Cu. To maintain adequate temperature, humidity and breathing conditions for the mice during irradiation, the plastic container was flushed with a constant air flow at a rate of 0.5 liter/min.

Pretreatment of Cells.—Spleen cells suspended in tissue culture medium were placed in sterile plastic tubes (Falcon 2001) at a concentration of 15–20 $\times$ 10^6 cells/ml and the pH adjusted in a gas flow of 10% CO_2, 7% O_2, and 83% N_2. The suspensions were warmed to 37°C in the incubator for 30 min before the addition of antigen and/or antibody. Preincubation with either antigen and/or antibody was terminated by cooling the tubes in ice and centrifuging them at 500 *g* for 7 min at 4°C. The cells were resuspended in cold tissue culture medium, washed four times before being suspended at a concentration of 15 $\times$ 10^6 cells/ml, and placed in tissue culture flasks with antigen.

Statistics.—Calculations of standard deviations, standard errors of the mean, *P* values according to Student's *t*-test, or the nonparametric rank test were performed by the University of Melbourne's IBM 7044 computer, using programs developed by Mr. V. Gledhill.

RESULTS

Suppression of the Primary Immune Response In Vitro by Passively Administered Antibody.—Antibody-mediated suppression of the immune response to

SRC in vitro has been reported by Mishell and Dutton (12). We have confirmed and extended this observation to show that the immune response to not only SRC, but also to POL could be specifically suppressed by the appropriate antiserum. To ascertain the specificity of the immune suppression by a given

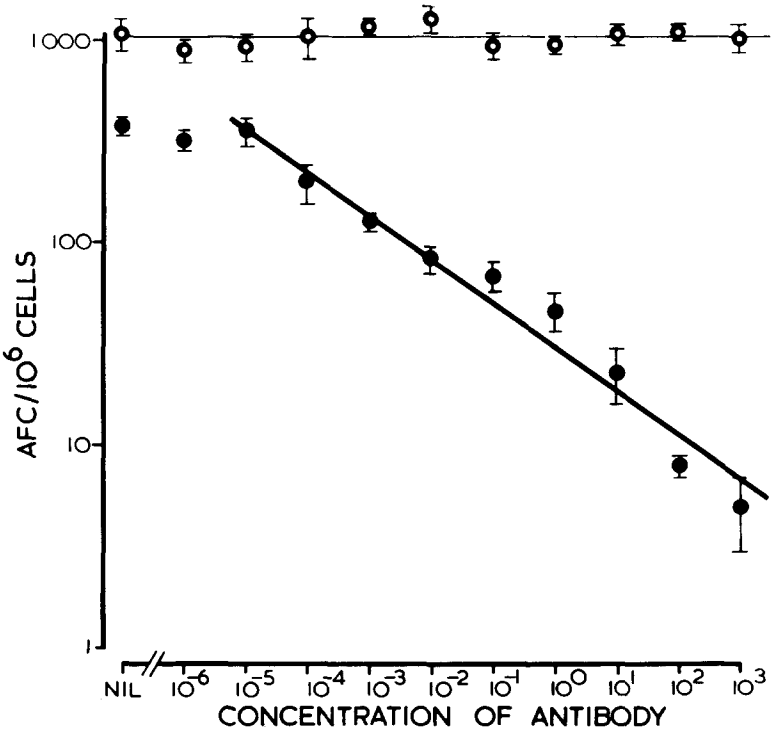

FIG. 1. Inhibition of the in vitro immune response to POL by anti-POL. Ordinate, number of antibody-forming cells to POL or SRC/10^6 harvested cells at day 4. Abscissa, reciprocal of antibody titer in culture flask. ●——●, immune response to POL of cultures exposed to anti-POL. ○——○, immune response to SRC of cultures whose response to POL was inhibited by anti-POL. Each point represents the arithmetic mean of four cultures ± the standard error of the mean.

antibody preparation, it was convenient to compare the results of spleen cell cultures immunized with both POL and SRC. Normal spleen cell suspensions which were immunized with both antigens in vitro responded as well as those immunized with only one antigen.

In experiments using serial dilutions of antisera, very small quantities of anti-POL inhibited the immune response of spleen cell cultures to 20 ng POL/ml. Using different pools of antiserum, the minimal concentration of anti-POL which suppressed the immune response varied between a theoretical titer of

10^{-2} and 10^{-4}. This represented the dilution of the original antiserum (titer 10^5), by a factor of between 10^7 and 10^9. The results of a single but typical experiment are given in Fig. 1, showing the full in vitro dose-response range of mouse spleen cell cultures to which serial dilutions of an antiserum were added at the begin-

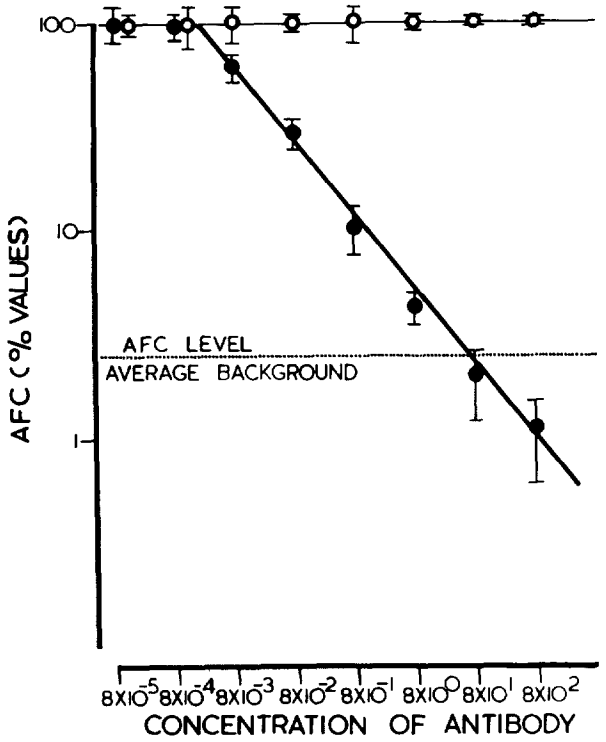

FIG. 2. Inhibition of the in vitro immune response to SRC by anti-SRC. Ordinate, number of antibody-forming cells to SRC at day 4, expressed as a percentage. Abscissa, reciprocal of antibody titer in culture flask. ●——●, immune response of cultures inhibited by anti-SRC. ○——○, immune response of cultures containing SRC only. ------, mean number of antibody-forming cells from cultures to which no SRC were added (background). Each point represents the arithmetic mean of 6–10 cultures ± the standard error of the mean.

ning of culture. It was found, from control cultures containing serum from non-immune mice, that high concentrations of normal mouse serum inhibited the in vitro immune response. Specific antiserum could therefore be used meaningfully only when its concentration was kept below that at which normal mouse serum becomes inhibitory. This restricted the dose of anti-POL to a titer of 10^3 or less (this represented 0.1 ml of a 1 in 10 dilution of the original antiserum, added to 0.9 ml of a spleen cell suspension containing antigen).

The effect of anti-SRC on the in vitro immune response of mouse spleen cells to 4×10^6 SRC/ml was also determined. Fig. 2 shows the pooled results from three such experiments. The responses of control cultures containing diluted normal mouse serum and SRC were taken as the equivalent of 100%. The other values were calculated by expressing the results obtained as a percentage of the response of control cultures. Very small doses of antiserum proved to be inhibitory, a hemagglutinin titer of 10^{-2} being at the limit of statistically significant inhibition in vitro.

In all subsequent experiments, the concentration of anti-POL was set at a titer of 10 in the culture flask, and that of anti-SRC at a titer of 8–10. With this concentration of anti-POL, the immune response to POL was markedly inhibited (90–95%), but could still be differentiated from the background, which was less than 0.1 AFC/10^6 spleen cells.

TABLE I

Suppression of the Background Response In Vitro by Anti-SRC

Treatment in vitro	Immune response
	AFC/10^6 cells ± SE of the mean
SRC	280.2 ± 61.6
No SRC (background)	16.3 ± 6.1
SRC and anti-SRC	2.8 ± 1.1*

* $P < 0.01$ as compared with all other groups. Each group represents the arithmetic mean of eight cultures ± the standard error of the mean.

Characteristically, the in vitro response to SRC exhibited a high background, due to cross-reacting antigens present in fetal calf serum (12). Using high titered anti-SRC, the in vitro immune response could be inhibited to a level lower than the background response (Table I).

Kinetics of Inhibition by Antibody Added at the Same Time as Antigen.— From a pooled spleen cell suspension cultures were set up containing 20 ng POL/ml. To one group anti-POL was added to give a final titer of 10, while the control group received anti-SRC of a similar dilution. Cultures were harvested after 1, 2, 3, and 4 days of incubation. The results of one such experiment are shown in Fig. 3 a. It can be seen that the two response curves are parallel, which suggests that the doubling time of AFC was the same in the presence as in the absence of anti-POL. This finding was verified in further experiments.

Cultures of spleen cells immunized with 4×10^6 SRC/ml, to which anti-SRC was added to a final titer of eight, were compared with immunized cultures containing an equal dilution of anti-POL. Cells were harvested at days 1, 2, 3, and 4. Results of a single but typical experiment are shown in Fig. 3 b. As found in

experiments using POL as the antigen, the doubling times, as deduced from the graph, were found to be the same whether anti-SRC was present or not.

Kinetics of Inhibition by Antibody Added after Antigen.—

Antibody was administered at various times after addition of the antigen to spleen cell cultures immunized with both SRC and POL. The antiserum was added in a volume of 0.1 ml and

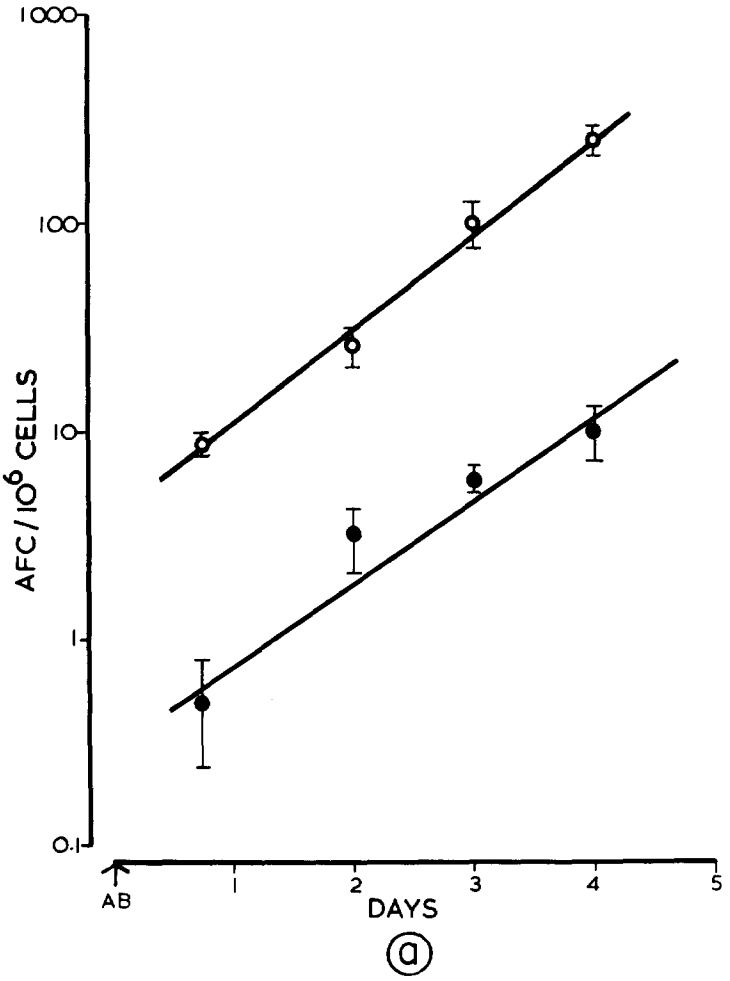

FIG. 3 a. Kinetics of the in vitro immune response to POL, inhibited by anti-POL. Antiserum was added at the commencement of culture. Ordinate, number of antibody-forming cells to POL/10^6 cells harvested. Abscissa, time from commencement of culture at which the cultures were tested for antibody-forming cells. ●——●, immune response of cultures inhibited by anti-POL. ○——○, immune response of cultures to which an antiserum of different antigenic specificity was added. Each point represents the arithmetic mean of eight cultures ± the standard error of the mean.

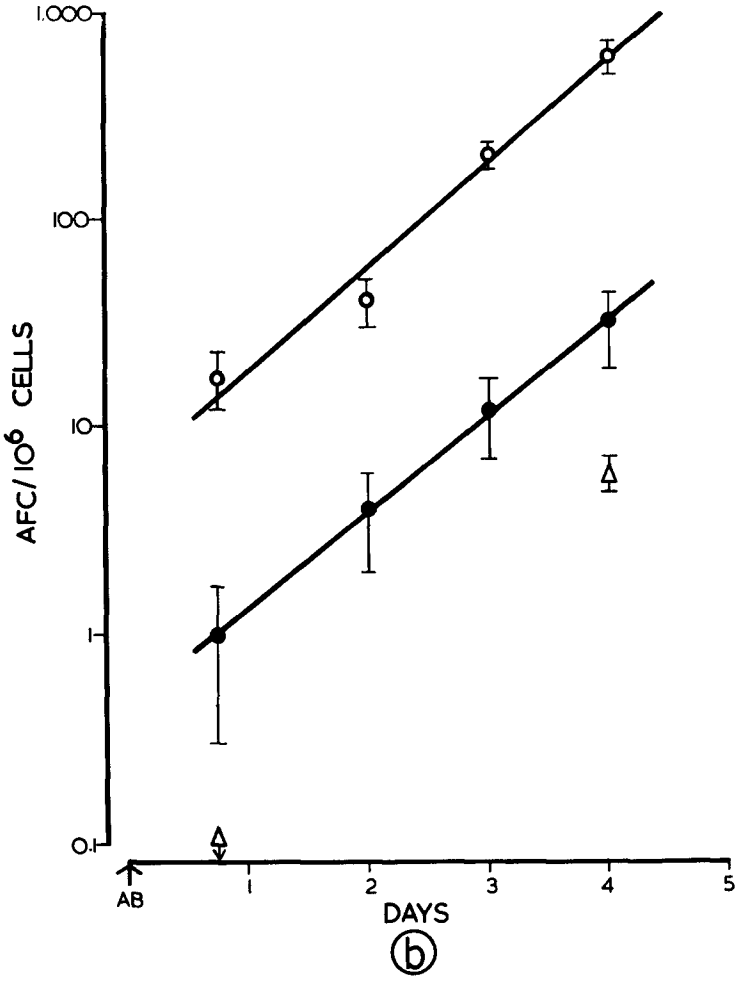

FIG. 3 b. Kinetics of the in vitro immune response to SRC, inhibited by anti-SRC. Antiserum was added at the beginning of culture. Ordinate, number of antibody-forming cells to SRC/10^6 cells harvested. Abscissa, time from commencement of culture at which cultures were tested for antibody-forming cells. ●——●, immune response of cultures inhibited by anti-SRC. ○——○, immune response of cultures to which an antiserum of irrelevant antigenic specificity was added. △, immune response of cultures to which no SRC were added. The arrow indicates that the value was smaller than the position on the graph may indicate. Each point represents the arithmetic mean of four cultures ± the standard error of the mean.

care was taken not to disturb the cells on the dialysis membrane of the culture flask. Mosier (25) has shown that if spleen cells cultured in vitro together with SRC are mechanically disturbed, no further increase in the number of AFC occurs. In order to distinguish between such a nonspecific effect and the specific effect of antibody, anti-SRC or anti-POL was added to cultures immunized with both SRC and POL.

The results of two such experiments are presented in Figs. 4 and 5. The specificity of inhibition by the added antiserum was verified by the suppression of the immune response to one antigen only.

The effect of passive antibody given after the cultures were immunized was

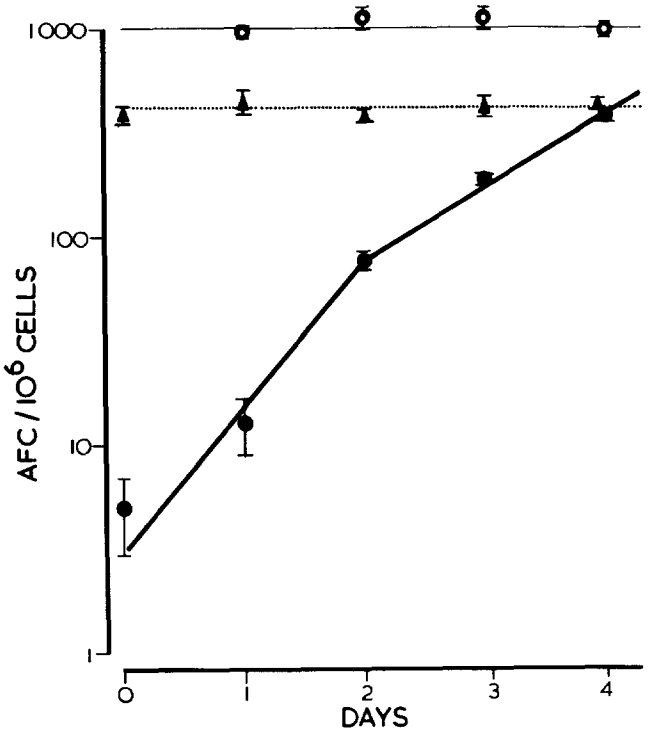

FIG. 4. Inhibition of the in vitro immune response to POL by anti-POL added subsequent to the antigen. Ordinate, number of antibody-forming cells/10^6 harvested cells at day 4. Abscissa, day after commencement of culture on which antiserum was added. ●——●, immune response to POL of cultures to which anti-POL was added. ▲. . . . ▲, immune response to POL of cultures to which anti-SRC was added. ○——○, immune response to SRC of cultures to which anti-POL was added. This graph illustrates results from a single but typical experiment. Each point represents the arithmetic mean of four to eight cultures ± the standard error of the mean.

apparent within 24 hr. This is unlike the situation in vivo, where a 2 day delay was observed (6). The suppressive effect of passive antibody was evident throughout the entire culture period.

The Immune Response to Various Concentrations of Antigen in the Presence of a Fixed Dose of Antibody.—The "peripheral" theory of antibody-mediated suppression suggests that antibody neutralizes antigen by covering up its deter-

minants. Immunocompetent cells are thus not stimulated because there is insufficient free antigen. According to this theory, the addition of more antigen, in the presence of a constant dose of antibody, should result in the initiation of an immune response, once antigenic excess is reached. The effect of increasing doses of POL in the presence of a fixed dose of anti-POL (titer 10) is illustrated in Fig. 6, which represents the pooled data from two experiments. It can be seen that the immune response was uniformly suppressed by anti-POL over the dose range from 2 ng to 30 μg POL/ml, in cultures which all concurrently gave a normal immune response to SRC. The lack of an immune response in cultures

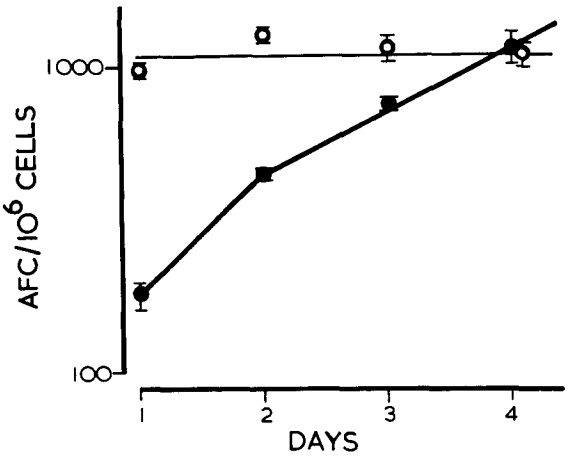

FIG. 5. Inhibition of the in vitro immune response to SRC by anti-SRC added subsequent to the antigen. Ordinate, number of antibody-forming cells to SRC/10^6 cells harvested at day 4. Abscissa, day after commencement of culture on which antiserum was added. ●——●, immune response to SRC of cultures to which anti-SRC was added. ○——○, immune response to SRC of cultures to which anti-POL was added. Each point represents the arithmetic mean of four to eight cultures ± the standard error of the mean.

exposed to 30 μg POL/ml suggested that antigenic excess may not yet have been achieved. To explore this further, spleen cells were cultured with 30 μg POL/ml and anti-POL (titer 10) for 6 hr. The supernatant was removed by centrifugation and tested for immunogenicity in vitro. Fresh spleen cells which were suspended in this supernatant diluted 10 and 100 times, respectively, gave a good immune response as shown in Table II. Thus the lack of response in spleen cells incubated in the presence of anti-POL and 30 μg POL/ml was due to factors other than lack of immunogenicity of POL in culture.

The Immune Response to Antigen-Antibody Complexes In Vitro.—The peripheral effect of passive antibody was further investigated by assessing the immunogenicity of complexes of antigen and antibody, made in antibody excess.

Such a study in vitro has the advantage that immune complexes are catabolized to a lesser extent than in vivo.

Complexes of POL and anti-POL were prepared by allowing 600 μg POL to react with 1 ml of anti-POL (titer 10^5) for 1 hr at 37°C, followed by 12 hr at 20°C. A visible precipitate was formed, which could be spun down. The supernatant after such interaction had an anti-POL titer of more than 10^2, indicating that the precipitate had formed in the zone of antibody ex-

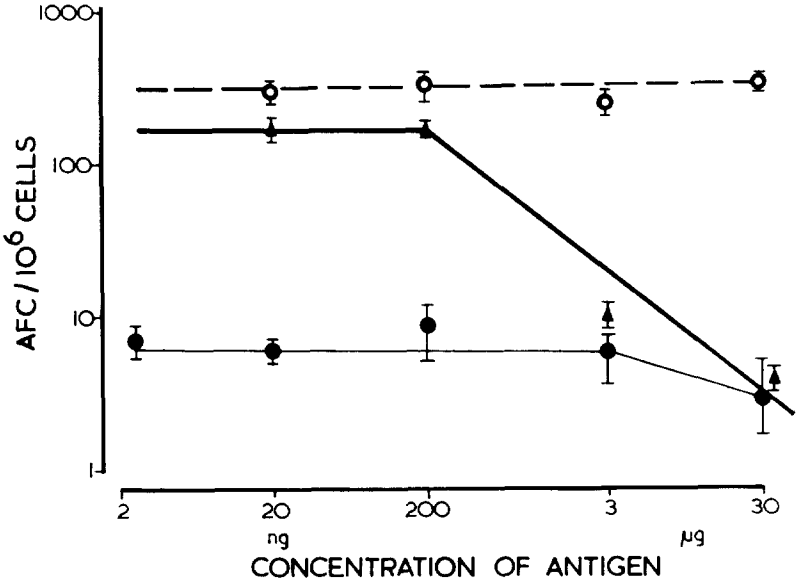

FIG. 6. The in vitro immune response to various doses of POL in the presence of a constant concentration of anti-POL. Ordinate, number of AFC/10^6 harvested cells at day 4. Abscissa, concentration of POL. ●——●, immune response to POL in the presence of anti-POL. ▲——▲, immune response to POL. ○——○, immune reponse to SRC of cultures whose response to POL was inhibited by anti-POL. The data illustrated were pooled from two experiments. Each point represents the arithmetic mean of eight cultures ± the standard error of the mean.

cess. The precipitate was washed five times in tissue culture medium using sterile technique. The supernatants left after the centrifugation of the complexes were kept and titrated. There was no detectable anti-POL titer after the third wash. The complexes were added in various amounts to cultures of spleen cells, and the immune response obtained was compared with that of cultures exposed to various concentrations of POL.

The results of this experiment are shown in Fig. 7. A normal immune response was elicited by the washed complexes at a concentration equivalent to 200 ng POL/ml. It was interesting to note that even *tolerance* to POL could be induced by the antigen-antibody complexes, in cultures which concurrently gave a normal response to SRC.

Preincubation of Spleen Cells with Antigen and Antibody In Vitro for Short Periods of Time.—Being unable to demonstrate a peripheral effect of passive antibody in vitro, the alternate hypothesis of a central mechanism was explored. Because POL is a soluble antigen, it can be washed free from spleen cells, and the duration of exposure to POL and anti-POL can be limited. Thus, provided that the state of suppression, once established, is at least temporarily irreversible, a central effect is potentially demonstrable.

Experiments were carried out in which normal mouse spleen cells at a concentration of 15×10^6/ml were exposed to a mixture of POL (20 ng/ml) and anti-POL (titer 10) at 37°C for various lengths of time. The cells were washed four times and subsequently cultured in the presence of both POL and SRC for 3 days. Control groups included spleen cells incubated either with anti-POL alone, with an antiserum of different antigenic specificity (anti-SRC), or with POL alone. Before adding the various agents, the pH of the cell suspension was adjusted by exposure to a gas flow of 10% CO_2, 7% O_2, and 83% N_2, and the temperature of the cell suspension raised to 37°C. The incubation procedure was terminated by rapid chilling of the cells in ice and centrifugation at 4°C as described under Methods. After adequate washing the cells were resuspended at a concentration of 15×10^6/ml, and transferred together with antigen to tissue culture flasks.

TABLE II

Immunogenicity of Supernatants from Spleen Cell Cultures Incubated for 6 hr with POL and Anti-POL

Experimental group	Immune response
	AFC/10^6 cells ± SE of mean
A. POL and anti-POL	5.0 ± 1.3
B. Supernatant	8.5 ± 3.4
C. Supernatant diluted 10 times	21.5 ± 3.1
D. Supernatant diluted 100 times	68.0 ± 17.1*
E. POL	101.8 ± 25.2

A: Spleen cells incubated for 6 hr with POL (30 μg/ml) and anti-POL (titer 10^1), washed and resuspended with POL (20 ng/ml).

B–D: Spleen cells incubated with different dilutions of the supernatant from group A.

E: Spleen cells incubated with POL.

There were 15×10^6 spleen cells/ml in each culture. All cultures gave a normal response to SRC. The duration of culture was 3 days.

* $P > 0.1$ as compared with group E.

$P < 0.01$ as compared with groups A and B.

Each value is the arithmetic mean of four to eight cultures ± the standard error of the mean.

Fig. 8 illustrates the immune response of such cultures harvested after 3 days of incubation. The data from three experiments have been standardized; the results from controls which had been incubated for a few minutes in the absence of any antiserum or in the presence of an antiserum of irrelevant spec-

ificity were taken as 100%. Such standardization proved to be necessary because of differences in the immune response of the controls due to the experiments being carried out with different batches of fetal calf serum. The immune response of all cultures to SRC was assayed and always found to be normal.

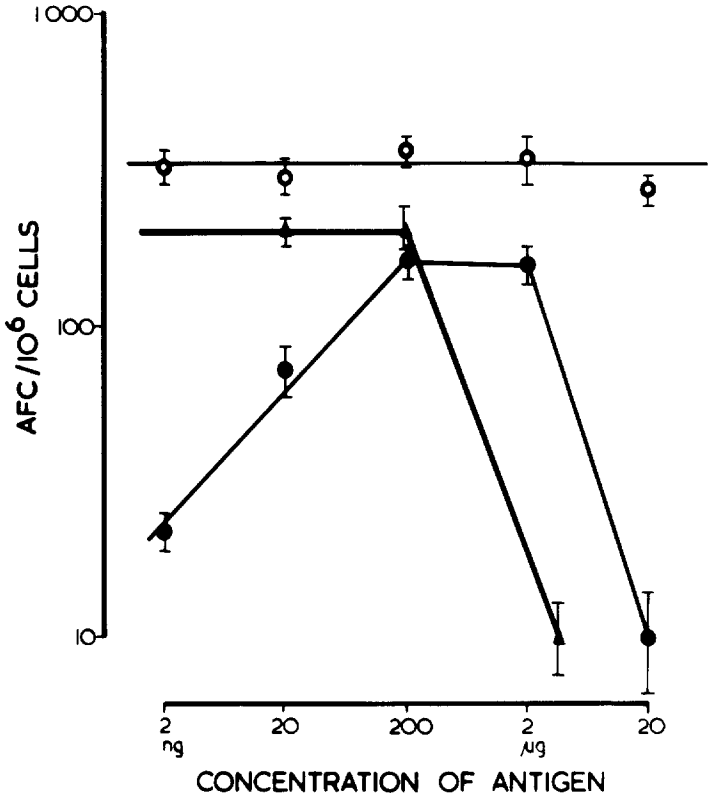

FIG. 7. The in vitro immune response to POL anti-POL complexes. The cultures were harvested at day 4. ●——●, immune response to POL anti-POL complexes. The concentration shown in the abscissa is the concentration of POL in the complexes. ▲——▲, immune response to POL. ○——○, immune response to SRC of cultures exposed to POL anti-POL complexes. Each point represents the arithmetic mean of five cultures ± the standard error of the mean.

The most important feature of these experiments was that normal spleen cells incubated with POL in the presence of anti-POL for short periods of time responded poorly to a subsequent in vitro challenge with an optimally immunogenic dose of POL. Inhibition of the immune response was apparent after preincubation of cells in the presence of POL and anti-POL for as short a period as 15 min. A further feature of these experiments was that incubation with antibody alone (Fig. 8) had no significant effect on the immune response.

These findings indicate that the suppressive action of antibody in combination with antigen was indeed acting at the level of the immunocompetent cell. It could, however, be argued that carry-over of antibody into the subsequent culture caused the immune suppression. There is much evidence against the latter view:

(*a*) Cells cultured with antibody alone gave a substantially normal response. If carry-over was due to nonspecific adsorption of proteins onto cells, then there

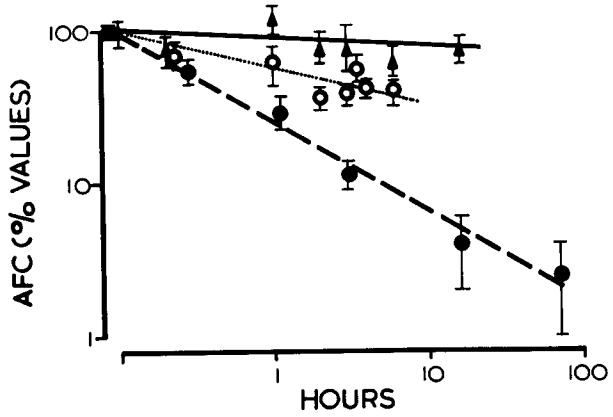

FIG. 8. The effect of preexposure of spleen cells to POL and anti-POL on the subsequent immune response to POL. Cultures were harvested at day 3. Ordinate, number of AFC expressed as a percentage of controls. Abscissa, hours of preincubation before washing. ●– – –●, immune response of cells incubated with POL and anti-POL. O . . . O, immune response of cells incubated with anti-POL alone. ▲——▲, immune response of cells incubated with anti-SRC or diluted normal CBA mouse serum. The data illustrated were pooled from three experiments and standardized so that controls incubated for a few minutes with anti-SRC or with normal mouse serum were taken as 100%. Each point represents the arithmetic mean of 6–12 cultures ± the standard error of the mean.

should have been as much carry-over (and hence immune suppression) in the presence or absence of POL.

(*b*) The degree of carry-over of antibody was estimated by the use of a radiolabeled anti-POL immunoglobulin preparation. The amount of radioactivity retained by the spleen cells after incubation and washing in conditions identical to those used previously is shown in Table III. By this method the carry-over of antibody after four washes was estimated to be 0.2–0.6%, a concentration which would not have been sufficient to cause the degree of inhibition detected. It is important to note that the carry-over of anti-POL (as judged by transfer of radioactivity) does not correlate with the subsequent immune response. The carry-over of antibody was not altered by the presence or absence of POL, but immune suppression occurred only if *both* POL and anti-POL were present during the period of incubation. Estimation of the carry-over of anti-POL was

also made by keeping the supernatants of cultures preincubated with antibody and/or antigen, and concentrating them 20-fold by negative pressure dialysis. No detectable titers were found in the supernatants obtained from the third and subsequent washes. Transfer of antibody into subsequent culture as estimated by this means was thus less than 1% of the initial concentration.

(*c*) The biological activity of the antibody carried over into subsequent culture was measured by mixing spleen cells that had been inhibited by incubation with antigen and antibody and subsequently washed, with normal spleen cells, and by culturing the mixture with antigen. By using both POL and SRC as

TABLE III

Influence of the Washing Procedure on the Carry-Over of Antibody after Preincubation of Cells with Radiolabeled Anti-POL and POL

Treatment	Time of exposure to ^{125}I anti-POL	^{125}I anti-POL carry-over after:				
		1st wash	2nd wash	3rd wash	4th wash	5th wash
		%	%	%	%	%
Anti-POL	5 sec	1.6	0.30	0.22	0.18	0.12
Anti-POL and POL	"	1.8	0.28	0.26	0.20	0.14
Anti-POL	15 min	1.85	0.33	0.30	0.29	0.16
Anti-POL and POL	"	1.90	0.27	0.19	0.17	0.12
Anti-POL	2 hr	1.83	0.93	0.62	0.60	0.51
Anti-POL and POL	"	1.81	0.58	0.52	0.43	0.33
Anti-POL	3 hr	1.21	1.33	0.19	0.16	0.12
Anti-POL and POL	"	1.27	0.58	0.28	0.22	0.20
Anti-POL	4 hr	2.0	1.12	0.92	0.43	0.31
Anti-POL and POL	"	1.92	0.72	0.67	0.60	0.42
Anti-POL	6 hr	2.6	1.12	0.93	0.43	0.30
Anti-POL and POL	"	1.8	0.81	0.72	0.30	0.34

Treatment of cells was carried out at 37°C. After each wash the cells were resuspended in large quantities of medium. The data were pooled from three separate experiments.

antigens, it was found (Tables IV and V) that normal spleen cells incubated in the presence of antibody-suppressed cells and antigen gave as good an immune response as the relevant controls.

(*d*) The kinetics of inhibition initiated by incubation of spleen cells with antigen and antibody revealed an exponential time course (Fig. 8). There was no significant inhibition if the cells were washed immediately after the addition of antigen and antibody. If washing was delayed by 15 min, there was a 40% inhibition of the immune response ($P < 0.05$), whereas after a delay of 5 hr, the immune response of the washed cells was diminished by 90% relative to that of the controls.

Preincubation of mouse spleen cells with antibody was also performed using SRC as antigen. Representative results are presented in Table VI. The immune

TABLE IV

Immune Response to POL of Normal Mouse Spleen Cells Cultured in the Presence of Antibody-Suppressed Cells

Experimental group	Number of cells	Immune response
	$\times 10^6$	*AFC/15 × 10^6 total cells cultured ± SE of mean*
A. Control (normal cells)	15	550 ± 78
	30	570 ± 52
B. Suppressed cells (6 hr in the presence of POL and anti-POL)	15	85 ± 8
C. Suppressed cells and normal cells	15 + 15	306 ± 25*

* The response of group C was not significantly different from that of group A, provided that the difference in the number of normal cells represented by 15 × 10^6 total cells in each group was taken into account.

Each value represents the arithmetic mean ± the standard error of the mean of four cultures. The results are expressed as AFC/15 × 10^6 total cells (normal or suppressed). The period of culture was 3 days. All cultures gave a normal response to SRC.

TABLE V

Immune Response to SRC of Normal Mouse Spleen Cells Cultured in the Presence of Antibody-Suppressed Spleen Cells

Experimental group	Number of cells	Immune response
	$\times 10^6$	*AFC/15 × 10^6 total cells cultured ± SE of mean*
A. Control (normal cells)	15	566 ± 149
	30	782 ± 72
B. Background (normal cells, no SRC)	30	55 ± 4
C. Suppressed cells (6 hr in presence of SRC and anti-SRC)	15	78 ± 10
D. Suppressed cells and normal cells	15 + 15	468 ± 80*

* Not significantly different from group A.

Each value represents the arithmetic mean ± the standard error of the mean of four cultures. The results are expressed as AFC/15 × 10^6 total cells (normal or suppressed). The period of culture was 3 days. All cultures gave a normal immune response to POL.

response of spleen cells preincubated with both SRC and anti-SRC was markedly diminished when compared to that of cells incubated in vitro with anti-SRC alone, with anti-POL alone, or with both anti-POL and SRC. These results are analogous to those found in the POL system. However, there is a potential difference in the experiments using POL as antigen and those using SRC. In

TABLE VI

Suppression of the In Vitro Immune Response to SRC by Exposure to SRC and Anti-SRC for 6 Hr

Treatment in vitro	Immune response
	AFC/10^6 cells ± SE of the mean
SRC for 4 days	392.3 ± 50.2
SRC and anti-SRC for 6 hr	77.8 ± 3.8*
Anti-SRC for 6 hr	462.5 ± 45.4‡
SRC and anti-SRC for 4 days	32.0 ± 8.1
No SRC (background)	12.0 ± 2.6

This table represents the results of a single but typical experiment. Each group represents the arithmetic mean of four cultures ± the standard error of the mean.

* $P < 0.05$ as compared with all other groups.

‡ Not significantly different to group incubated with SRC.

TABLE VII

Suppression Induced In Vitro of the Adoptive Immune Response of Mouse Spleen Cells to POL

Experiment No.	Treatment in vitro	Number of mice	Number of viable cells transferred	Immune response		
				Specific activity	ARC foci	Serum titer
E5	Anti-SRC	8	5×10^6	37.9 ± 3.1	3.86 ± 0.76	—*
	Anti-POL and POL	9	"	6.6 ± 2.3‡	0.78 ± 0.22‡	—
	Anti-POL	6	"	39.5 ± 11.1	2.88 ± 0.79	—
	POL	7	"	71.4 ± 11.3§	4.67 ± 0.67	—
E4	Anti-SRC	8	4×10^6	44.1 ± 7.9	3.8 ± 0.8	-
	Anti-POL	8	"	31.9 ± 6.7‖	3.2 ± 0.7‖	—
E6	Normal mouse serum	9	5×10^6	49.1 ± 13.3	3.00 ± 0.73	30.8 ± 1.5
	Anti-POL and POL	5	"	9.4 ± 6.1‡	1.00 ± 0.63‡	10.0 ± 1.2‡
	Anti-POL	5	"	32.0 ± 11.3	2.20 ± 0.49	52.5 ± 1.4
E9	Anti-SRC	14	2×10^6	20.7 ± 3.4	2.57 ± 0.42	19.7 ± 1.2
	Anti-POL and POL	18	"	6.5 ± 2.4‡	0.67 ± 0.22‡	3.2 ± 1.4

The specific activity and the ARC foci are expressed as the arithmetic mean ± the standard error of the mean. The serum titers are expressed as the geometric mean ± the standard error of the mean.

* —, not determined.

‡ $P < 0.01$ as compared with all other groups.

§ $P < 0.05$ as compared with the group incubated with anti-SRC.

‖ $P > 0.1$ (not significant).

At cell doses of 4×10^6 cells and 5×10^6 cells there is confluence of the ARC foci and hence the numbers of foci in the controls are reduced below the true values. Background responses (of mice not injected with cells) have not been subtracted.

experiments using SRC as antigen, the carry-over of antibody may be much greater than in experiments performed with POL. SRC are particulate and sediment with spleen cells on centrifugation. Thus, antibody adsorbed onto SRC cannot be washed away, and can be transferred into subsequent cultures or into irradiated mice.

Content of Immunocompetent Cells Reactive to POL in Mouse Spleen Cell Suspensions Exposed to Antigen and Antibody In Vitro.—

To test the effect of antibody-mediated suppression at the level of the immunocompetent cell, normal mouse spleen cell suspensions were exposed to anti-POL and POL in vitro for 12 hr

TABLE VIII

Suppression Induced In Vitro of the Adoptive Immune Response of Mouse Spleen Cells to SRC

Experiment No.	Treatment in vitro	Number of mice	Number of viable cells transferred	Immune response		
				Specific activity	ARC foci	Serum titer
E6	Normal mouse serum	9	5×10^6	30.3 ± 6.2	4.3 ± 0.63	17.5 ± 1.3
	SRC and anti-SRC	9	"	11.4 ± 5.0*	1.3 ± 0.37	3.9 ± 1.6*
	Anti-SRC	5	"	19.2 ± 4.5	3.2 ± 1.1	10 ± 1.2
	SRC	5	"	68.5 ± 13.3	3.5 ± 0.65	52.5 ± 1.6
E8	Anti-POL	14	2×10^6	21.0 ± 3.7	2.6 ± 0.33	17.0 ± 1.5
	SRC and anti-SRC	10	"	3.2 ± 2.6*	0.30 ± 0.21*	1.2 ± 1.2*
	Anti-SRC	14	"	33.8 ± 8.4	2.15 ± 0.25	10.7 ± 1.4

The specific activity and the ARC foci are expressed as the arithmetic mean ± the standard error of the mean. The serum titers are expressed as the geometric mean ± the standard error of the mean.

* $P < 0.01$ as compared to all other groups.

Background responses (of mice not injected with cells) have not been subtracted.

before harvesting. The cells were washed four times and injected intravenously into lethally irradiated syngenic mice. Control groups included cells exposed to anti-POL alone, to POL alone, to an irrelevant antiserum (anti-SRC), or to normal CBA mouse serum in the appropriate dilution. Recipient mice in all the groups were challenged with 25 μg POL.

The results of four such experiments are given in Table VII. A reduction in the number of immunocompetent cells was repeatedly found in cells cultured in the presence of both POL and anti-POL. This is in contrast to the normal response of cells incubated with anti-POL alone. Cells incubated with POL alone gave a significantly increased response as compared to cells cultured without POL, thus indicating that "priming" had occurred within the 12 hr of culture in vitro.

The immune status of mice which had received cells incubated in vitro with

both POL and anti-POL was also assessed by another parameter, the serum titer. In some experiments (Table VII) mice were exsanguinated under chloroform anaesthesia prior to splenectomy. Results illustrated in Table VIII show that the serum titer was diminished in close parallel to the reduction in the number of ARC foci. Thus, the diminution of ARC foci detected was a true reflection of the immune status, and not merely due to an altered seeding efficiency of immunocompetent cells to the spleen. The differences in all the

TABLE IX

Suppression Induced In Vivo of the Adoptive Immune Response of Mouse Spleen Cells to POL

Experiment No.	Treatment in vivo	Number of mice	Number of viable cells transferred	Immune response		
				Specific activity	ARC foci	Serum titer
E14	Anti-SRC	8	2×10^6	77.6 ± 10.2	3.3 ± 0.62	21.4 ± 2.2
	POL and anti-POL	7	"	12.5 ± 4.5*	0.87 ± 0.39‡	1.8 ± 1.3*
	Anti-POL	10	"	45.3 ± 11.0	3.14 ± 0.26	37.2 ± 1.2
	POL	5	"	24.5 ± 10.9	1.75 ± 0.48	12.2 ± 1.4
E7	Normal mouse serum	10	5×10^6	91.6 ± 3.6	§	—‖
	POL and anti-POL	10	"	45.2 ± 12.2*	§	—
	Anti-POL	6	"	86.1 ± 2.9	§	—

The specific activity and the ARC foci are expressed as the arithmetic mean ± the standard error of the mean. The serum titers are expressed as the geometric mean ± the standard error of the mean.

* $P < 0.05$ as compared with all other groups.

‡ $P < 0.01$ as compared with anti-SRC, anti-POL.

§ Confluence; groups 1 and 3 were composed mostly of spleens with all the slices positive.

‖ —, not determined.

Background responses (of mice not injected with cells) have not been subtracted.

immune parameters measured (ARC foci, specific activity, and serum titer) were significant ($P < 0.01$) when compared with the control groups. To exclude the possibility that the diminution of the number of ARC foci was due to transfer of anti-POL with the spleen cells into the irradiated host the effect of anti-POL (in the concentration used in tissue culture) on the response to 25 μg POL of irradiated mice repopulated with 5×10^6 spleen cells was tested. When assayed at day 7, mice injected with anti-POL had 822 ± 210 AFC/spleen, whereas mice injected with normal CBA serum had 1060 ± 302 AFC/spleen. The concentration of anti-POL, which was found to be without suppressive effect, was as much as would have been transferred if the cultured cells were not washed at all. Clearly then, the immune suppression detected in vivo was not a result of the carry-over of antibody.

The inhibition of the immune response of spleen cells to SRC by anti-SRC induced in vitro was also analyzed at the level of the antigen-reactive cell. The results are presented in Table VIII. The data obtained with POL were confirmed. The most important results were the specific reduction in the number of ARC foci and of the serum titer of mice receiving cells from cultures containing both SRC and anti-SRC.

Suppression of the Primary Immune Response In Vivo.—

Splenic content of immunocompetent cells reactive to POL or SRC: Preliminary experiments had shown that the primary response of CBA mice given 25 μg POL intraperitoneally and

TABLE X

Suppression Induced In Vivo of the Adoptive Immune Response of Mouse Spleen Cells to SRC

Experiment No.	Treatment in vivo	Number of mice	Number of viable cells transferred	Immune Response		
				Specific activity	ARC foci	Serum titer
E13	Anti-POL	6	2×10^6	19.3 ± 7.6	1.83 ± 0.40	8.9 ± 1.3
	SRC and anti-SRC	6	"	2.8 ± 1.2*	0.50 ± 0.22*	2.2 ± 1.5‡
	Anti-SRC	5	"	12.9 ± 2.3§	1.40 ± 0.24§	6.6 ± 1.2§
	SRC	9	"	13.1 ± 4.1§	1.33 ± 0.37§	5.7 ± 1.3§
E16	Culture medium	10	2×10^6	19.1 ± 2.6	1.60 ± 0.22	5.6 ± 1.1
	SRC and anti-SRC	10	"	2.4 ± 1.7*	0.30 ± 0.15*	1.2 ± 0.4‡
	Anti-SRC	10	"	14.1 ± 3.0§	1.30 ± 0.21§	6.5 ± 1.5

The specific activity and the ARC foci are expressed as the arithmetic mean ± the standard error of the mean. The serum titers are expressed as the geometric mean ± the standard error of the mean.

* $P < 0.01$ as compared with all other groups.

‡ $P < 0.05$ as compared with groups exposed to anti-POL or culture medium only.

§ Not statistically significant as compared with group injected with anti-POL.

Background responses (of mice not injected with cells) have not been subtracted.

assayed at 4 days could be reduced 20–40-fold by a prior intravenous injection of 0.1 ml anti-POL (titer 10^4).

The above dose of anti-POL was given intravenously 3 hr prior to the intraperitoneal injection of 25 μg POL. In view of the in vitro findings described previously, it was decided to transfer spleen cells into recipient mice 12 hr after the administration of POL. In vitro, this period of exposure to POL and anti-POL resulted in a marked degree of immune suppression. Single cell suspensions were made and washed three times. Into each lethally irradiated mouse $2–5 \times 10^6$ viable cells were injected. Control groups included recipients given spleen cells from mice treated with POL alone, with anti-POL alone, and with either an irrelevant antiserum (anti-SRC) or with normal mouse serum. All recipients were challenged with 25 μg POL intraperitoneally 6–24 hr thereafter.

The results of two such experiments are recorded in Table IX. The most important feature was the significant reduction in the immune response, both

at the level of the immunocompetent cell (ARC) and in the serum titer, of recipient mice given cells which had been exposed in vivo to both antigen and antibody.

In order to estimate the degree of suppression caused by the anti-POL in the donor mice, two mice were kept in each group and assayed for their splenic content of AFC at day 4 (peak response). In experiment E7, mice given normal mouse serum prior to 25 μg POL had a mean response of 70,000 AFC/spleen, as compared with a mean of 2600 AFC/spleen in those mice given POL and anti-POL. Table IX shows that the immune suppression, as detected by cell transfer, is of a lesser degree (2–5-fold) than that of the donor mice (27-fold) used in the experiment cited. Table IX also shows the entirely normal response of cells from mice given anti-POL alone.

The inhibition of the immune response which could be induced in vivo by passive antibody was also measured at the level of the immunocompetent cell in the SRC system. The results in Table X are in agreement with those obtained with POL. Significant suppression of all the immune parameters tested was obtained. Mice of the same groups as those whose spleen cells were transferred in experiments described in Table X, were kept and assayed for their splenic content of AFC at day 4. The immune response of mice inhibited by anti-SRC was 30 times less than that of controls. In contrast, the immune response of the transferred spleen cells as measured at the level of the antigen reactive cell was only reduced about five-fold.

DISCUSSION

Recent work has shown that immunity and immunological tolerance may be induced in vitro to the protein H antigens of *S. adelaide* (14). This report deals with the study, in vitro, of another phenomenon of humoral immunity, that of antibody-mediated suppression of the immune response.

Passive antibody can prevent the initiation of an immune response, or shorten and diminish an already established primary response. The in vitro studies we have reported in the present paper are mainly concerned with specifically preventing the initiation of the immune response by the relevant antibody. It is not known whether the mechanisms of antibody-mediated suppression during immune induction are the same as those which act on an already established immune reaction.

Our work puts new emphasis on the hypothesis originally suggested by Rowley and Fitch (9), that the mechanism of inhibition of the early primary response by passive antibody occurs in the "central" part of the immune system by acting directly on the immunocompetent cells themselves. Experimental data cited in this report provide strong support for this concept.

The most important evidence for the hypothesis that passive antibody acts at the cellular level comes from the results of preincubation experiments.

Brief exposure to mixtures of antigen and antibody produced a significant reduction in the capacity of mouse spleen cells to respond to an antigenic challenge, either in vivo or in vitro. The kinetics of such immune suppression in vitro to POL follow an exponential time course, and closely parallel the kinetics of immune tolerance induced in vitro (11). A significant reduction in immune capacity was found to occur within 15 min of exposure of cells to antigen and antibody, while marked suppression (90%) required such treatment for 4–6 hr. This suggests that the immune suppression is due to a specific process of interaction of antigen and antibody molecules with the relevant cells, rather than to mere physical carry-over of antibody which would neutralize antigen added subsequently. This concept is further supported by critical tests in which the carry over of antibody was monitored. Neither was the quantity of antibody carried over sufficient to cause the degree of immune suppression that was found, nor did it depend on the presence or absence of antigen (Table III). This latter finding is highly relevant to the fact that the preincubation of cells with antibody in the absence of antigen failed to cause immunosuppression. The normal immune response of spleen cells cultured in the presence of antigen and antibody-suppressed cells verified that the immune suppression obtained was due to a direct effect on the immunocompetent cells.

Having found evidence for a central effect of antibody on lymphoid cells exposed to antigen in vitro, further direct proof of the existence of this phenomenon was obtained by testing the splenic content of immunocompetent cells of mice injected with both antigen and antibody. A significant reduction in the number of immunocompetent cells reactive to POL or SRC was induced by treatment with the appropriate antigen and antiserum in vivo.

The idea that antibody could directly reduce the number of immunocompetent cells reactive to the corresponding antigen was first enunciated by Rowley and Fitch (9). This hypothesis was suggested to explain the reduction in immunocompetence of rat spleen cells exposed to rat anti-sheep erythrocyte serum, either in vitro or in vivo. Because the presence of antigen together with antibody was not essential in their experiments, the basis for the strict antigenic specificity of suppression is hard to explain. Rowley and Fitch (26) postulated that immunocompetent cells ("potential antibody-forming cells") have receptors on their surfaces with the configuration of the antigenic determinants to which they may respond. The results which we have interpreted as providing evidence for a direct effect on antigen-reactive cells are of a somewhat different nature. We could induce specific nonresponsiveness only in the presence of both antigen and specific antibody, and thus the question of the strict specificity of immunosuppression is readily answered. Antigen may be required to focus the relevant antibody to the target cell. The recognition of antigen is presumably mediated by antigen specific recognition sites at the surface of the immunocompetent cell.

In studying the in vitro immune response to POL, experiments were performed to test directly for the presence of a peripheral mechanism of passive antibody. We could not demonstrate such a mechanism either by measuring the immune response to various concentrations of POL in the presence of a constant concentration of anti-POL, or by exposing spleen cells in vitro to washed complexes of POL and anti-POL. In the presence of a fixed concentration of anti-POL, the concentration of POL was increased to levels which were found to be in antigen excess, as verified by the supernatants being immunogenic. It would thus seem that anti-POL could be immunosuppressive in vitro, without being in a concentration sufficient to cover all the antigenic determinants. A similar effect was shown in vivo by Uhr and Baumann (1), where the primary immune response to tetanus toxoid was inhibited by a dose of antitoxin insufficient to cover all the antigenic determinants of the toxoid administered.

Dissociated spleen cells were capable of responding to washed complexes of POL and anti-POL made in antibody excess. In these experiments it could be assumed that the maximum number of antigenic determinants were covered by antibody. Despite this, a good immune response was elicited in vitro, not significantly different from that obtained with POL in optimal concentration. The possibility cannot be excluded that such immune responses were due to dissociation of the complexes. However, such dissociation could presumably occur in vivo, in antibody-suppressed animals. Our results were in contrast to the findings of other investigators, who have failed to elicit an immune response, in vivo or in vitro (9, 12, 30) to complexes of SRC and anti-SRC. Possibly the difference in the results obtained was due to the differing characteristics of the antigens, or the various methods used. One result obtained in vivo that would be hard to reconcile with the concept of antibody-mediated suppression occurring solely at the peripheral level, was that washed complexes of SRC and anti-SRC inhibited the subsequent immune response of rats to 10^9 SRC (30). Conceivably, this may be an example of tolerance induction by antigen-antibody complexes, as was shown with regard to another antigen in the rabbit (27).

In vivo, the doubling times of AFC in the primary immune response depended on the antigen dose (28–31). In contrast, the doubling times of AFC in antibody-suppressed animals were the same as those of AFC from animals which received the same amount of antigen only (30). Our results in vitro similarly show that the doubling times of AFC in antibody-suppressed cultures were the same as that of controls. This evidence again fails to support the peripheral theory of antibody-mediated suppression, which implies that the effect of doses of antibody which reduce but do not totally prevent the immune response is to lessen the magnitude of the antigenic stimulus. These results thus suggest that the cellular events underlying the antibody-suppressed immune response and the response to suboptimal antigen doses are not the same.

Passive antibody given after the initiation of the primary immune response in vivo did not have much effect for a period of 1–2 days, and then led to the premature cessation of the exponential phase of antibody production and to more rapid decay of the immune response (6). By contrast, the effect of passive antibody given at any time during the in vitro immune response was more quickly apparent, being detectable within 24 hr. A possible reason for this difference is that the distribution of passive antibody in vitro rapidly becomes uniform, and thus interaction with antigenic determinants would occur much more readily. In vivo, sequestration of antigen and antibody into different anatomical sites or intracellular compartments may occur. This may explain the increased latent period before the effect of passive antibody given late in the primary response becomes manifest. As in vivo, the later the antibody was administered, the less was the detectable degree of suppression.

Little is known of the molecular events occurring at the cell surface by which interaction of antigen and antibody can facilitate specific immunological unresponsiveness. The only detail that we know at present is that recognition of antigenic determinants is probably the first event occurring at the cell surface, since antigen is a prerequisite for immune suppression.

The immune suppression found after preincubation of spleen cells with mixtures of antigen and antibody is analogous to immune tolerance, being antigen-specific and involving a central unresponsiveness of the immune system. Furthermore, the kinetics of induction of unresponsiveness by POL and anti-POL and those of immune tolerance to POL in vitro (11) are closely parallel. It is possible that the direct effect of antibody and antigen on immunocompetent cells is due to the induction of tolerance by immune complexes, which may form on the surface of immunocompetent cells. A detailed analysis of the cellular mechanism of antibody-mediated immune suppression is the subject of a forthcoming communication. Studies of suppression of the immune response by antibody fragments in vivo have yielded conflicting results (26, 32–34), partly due to the increased catabolism of these fragments (35). The effect of antibody fragments on the immune response in vitro, without the drawback of increased catabolism, should be of interest.

SUMMARY

Antibody-mediated suppression of the in vitro immune response to polymerized flagellin of *Salmonella adelaide* and to sheep erythrocytes was studied at the cellular level. Normal mouse spleen cells, preincubated in vitro with mixtures of antigen and antibody for short periods of time before being washed, did not respond to an optimal antigenic challenge in vitro, whereas similar cells treated with antibody alone gave a normal response. The degree of immune suppression was found to depend on the time of preincubation. Significant immune suppression could be induced in as short a time as 15 min, whereas profound suppression (90%) required the incubation of cells with mixtures of

antigen and antibody for 4–6 hr. Mouse spleen cells treated similarly were also unable to respond subsequently to the antigen upon transfer to lethally irradiated hosts, as measured at both the level of the antigen-reactive cell and that of serum antibody production. These results were taken as evidence that in vitro an effect of antibody-mediated suppression occurred at the level of the immunocompetent cell.

Similarities between immune tolerance and antibody-mediated suppression in vitro were described, and the significance of the findings discussed in the light of current concepts of the mechanism of antibody-mediated suppression.

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