HOMEOSTASIS OF ANTIBODY FORMATION IN THE ADULT RAT*

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Passive immunization of rats with homologous anti-sheep erythrocyte serum markedly inhibits the primary antibody response to sheep erythrocytes. Furthermore, unresponsiveness to sheep erythrocytes induced by a single passive immunization is sustained by weekly injections of sheep erythrocytes (1). The mechanism producing sustained immunological unresponsiveness in adult animals by this simple procedure has not been elucidated.

Experiments to be reported in this paper suggest that specific antibody adsorbed onto or incorporated into potential antibody-forming cells inhibits these cells from forming specific antibody. Antibody does not have this effect on cells from actively immunized rats. Thus, the formation of specific antibody may provide a homeostatic or "feed-back" mechanism which controls or limits production of specific antibody to that portion of the antibody-forming system previously stimulated by the antigen. This mechanism may account in part for immunological unresponsiveness produced in mature animals by various procedures.

Materials and Methods

Young adult male Sprague-Dawley rats, weighing 180 to 250 gm were fed Purina chow and water *ad libitum*. All injections were in the lateral tail vein.

Antigens and Antisera.—The antigens were washed whole sheep erythrocytes (1) or flagella of *Salmonella typhosa* (2). Doses of erythrocytes, recorded as per cent suspension, were prepared from packed cells. The concentration of erythrocytes in suspensions was standardized by hemoglobin determinations. A 1 per cent suspension of sheep erythrocytes contained about 2×10^8 cells/ml. Cell counts were always made when dosages are reported as numbers of erythrocytes.

Pooled rat anti-sheep erythrocyte serum was obtained from a large group of rats exsanguinated 6 days after receiving a single injection of 1.0 ml of 0.25 per cent sheep erythrocytes. Pooled rat antiflagellar serum was obtained from rats exsanguinated 6 days after receiving 1.0 ml containing 10 μg protein of the flagellar antigen. The pooled sera were stored at −30°C

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until used. Antibody titrations against sheep erythrocytes or whole killed *S. typhosa* were carried out as previously described using double dilution technique (1, 2); the initial serum dilution was 1:10. The titers were recorded as the number of the last tube showing complete hemolysis of sheep erythrocytes or the number of the last tube containing grossly visible aggregations of *S. typhosa*; thus, the titers expressed the serum dilution as 10×2^n where n is the tube number. On repeated titrations the pooled anti-sheep erythrocyte serum had a titer of 7 to 8, and the pooled antiflagellar serum had a titer of 6 to 7. Mean titers for groups of rats ± the standard error of the mean are reported in Tables I to III; a titer of 0 was arbitrarily assigned to sera showing less than the end point reaction to the antigen at a serum dilution of 1:10.

In some experiments sera were treated with 2-mercaptoethanol (2-ME) for antibody titrations or for passive immunizations. For these titrations, sera diluted 1:5 in saline were added to an equal volume of 0.1 M 2-ME and incubated at 37°C for 30 minutes (3). As in the usual titrations, the initial serum dilution was 1:10 and the subsequent titration procedure was the same as for untreated sera. For passive immunizations, 10 ml of antiserum diluted 1:5 in saline was added to an equal volume of 0.1 M 2-ME and incubated at 37°C for 30 minutes; the mixture was restored to original volume using pressure dialysis in the cold. For convenience, the antibody inactivated by 2-ME will be referred to as "19S" antibody and the antibody not inactivated by 2-ME will be referred to as "7S" antibody.

Spleen Cells and X-Irradiated Recipients.—The capacity of spleen cells to form antibody was measured by transferring the cells to rats made unresponsive by x-irradiation. Spleens were minced and passed through a No. 80 tantalum gauze screen using a pestle and a modified tissue culture medium (4). The same medium was used for washing and diluting the spleen cells. Tissue fragments and large aggregates of cells were removed by low-speed centrifugation for 30 seconds. The spleen cells were washed twice. The number of nucleated cells was determined by hemocytometer counts. Spleen cells were prepared at room temperature. No immediate or late untoward effect was observed in any rat receiving the spleen cell suspensions. Irradiated rats received 600 roentgens total body x-irradiation given as a single dose. X-Rays were generated in a 250-KVP machine operating at 15 ma with 0.25 mm Cu and 1 mm Al added filtration. The dose rate averaged 39 rad per minute at 75 cm.

Plaque-Forming Cells and Plate Hemolysin Titers.—The method of Jerne and Nordin for demonstrating release of antibody by single cells in agar plates was used with minor modifications (5). Tissue culture medium (4) containing no serum was used for preparing spleen cell and sheep erythrocyte suspensions and agar plates. DEAE dextran was added to the agar solutions (6). Two ml of fresh normal rat serum diluted 1:3 in normal saline was used for complement. Plates were incubated for 1 hour at 37°C before addition of complement, and for 1 hour after addition of complement. Plates were rinsed in normal saline at the completion of incubation and were counted immediately using magnification of 10 to 25 and transillumination of the plates.

Each rat spleen was minced and passed through a No. 80 tantalum gauze screen using a pestle and 4.0 ml of tissue culture medium. The volume was brought to 5.0 ml and tissue fragments and large aggregates of cells were removed by low-speed centrifugation for 30 seconds. The number of nucleated cells in the suspension was determined by hemocytometer counts. Plates were prepared in triplicate from the suspension, and from 3 fivefold serial dilutions of the suspension. The total number of plaque-forming cells per spleen or per 10^6 recovered spleen cells was calculated from the average plaque count of at least 3 plates.

The agar plate technique was sufficiently sensitive to demonstrate hemolytic antibody released by single cells. Quite reasonably, agar plates containing a thin layer of sheep erythrocytes could be used as a sensitive method for demonstrating anti-sheep erythrocyte antibody in serum. Agar plates were prepared as for demonstrating plaque-forming cells but only sheep

erythrocytes were added to the agar in the thin overlayer. Microdroplets of about 0.005 ml of undiluted inactivated serum and of serial twofold dilutions of the serum were spotted on the plate. The plates were incubated at 37°C for 30 minutes, then rinsed several times with saline. Two ml of fresh rat serum diluted 1:3 in saline was added as complement, and the plates were incubated again at 37°C for 30 minutes. Low dilutions of anti-sheep erythrocyte serum produced clear "plaques" of complete hemolysis about 2 to 3 mm in diameter which were considered a 4+ reaction. At higher serum dilutions, distinct plaques of partial hemolysis occurred which could be arbitrarily ranked as 3+ to 1+ reactions. Since only small volumes of serum were required, it was possible to begin titrations with undiluted serum.

Histological Preparations.—Multiple 1 mm-thick transverse slices of spleen were fixed for 2 hours in Carnoy's alcohol–acetic acid-chloroform mixture. Tissues were embedded in paraffin, sectioned at 5 microns, and stained with methyl green–pryonine (7).

PRELIMINARY EXPERIMENTS

The Disappearance of Passively Transferred Antibody.—A total of 28 rats injected with 1.0 ml of the anti–sheep erythrocyte serum received no other injections and were bled 1, 4, and 6 days later. At 1 day the mean titer for all rats was 2.3 (range of 1 to 3), at 4 days the mean titer was 0.3 (range of 0 to 1), and at 6 days the mean titer was 0. The half-life of circulating passively transferred antibody was about 1.5 days. Thus, in subsequent experiments, antibody titers of sera obtained 5 or more days after both passive and active immunization against sheep erythrocytes measured actively formed antibody.

Inhibition of the Antibody Response to Various Doses of Erythrocytes.—Rats passively immunized with 1.0 ml injections of anti-sheep erythrocyte serum were injected 24 hours later with 1.0 ml of 0.25, 2 or 4 per cent sheep erythrocytes. Non-passively immunized rats (controls) were similarly injected with the same doses of sheep erythrocytes. The mean 6 day titers for the passively immunized rats were 0.4, 1.0, and 1.5 and for the control rats 8, 7.5, and 8.2. Titers for all rats were lower at 10 and 14 days. Thus, the determination of titers at 6 days, the time of peak antibody response, accurately demonstrated inhibition of antibody formation produced by passive immunization.

Specificity of Inhibition of Antibody Formation.—Rats, passively immunized with either anti–sheep erythrocyte serum or with anti–*S. typhosa* serum, were actively immunized 24 hours later with sheep erythrocytes or with flagellar antigen. The anti–sheep erythrocyte serum inhibited the antibody response to sheep erythrocytes but not the response to *S. typhosa*. Similarly, the antiflagellar serum inhibited the agglutinin response to *S. typhosa* but not the response to sheep erythrocytes.

Inhibition of the Antibody Response by "19S" or "7S" Antibody.—The pooled anti–sheep erythrocyte serum had a titer of 7 to 8 and after treatment with 2-ME no measurable titer at a dilution of 1:10, indicating that the serum contained predominantly 19S antibody. Passive immunization of rats with the antiserum treated with 2-ME did not suppress the antibody response to sheep erythrocytes.

A second pool of serum obtained from rats which had received multiple injections of sheep erythrocytes had a titer of 8 and after a treatment with 2-ME a titer of 6, indicating that the serum contained a mixture of 19S and 7S antibody. Five rats were passively immunized with 1.0 ml injections of the serum containing a mixture of 19S and 7S antibody; 5 rats were passively immunized with 1.0 ml of the same serum treated with 2-ME and containing predominantly 7S antibody, and a third group of 5 animals was passively immunized with the primary response antiserum containing predominantly 19S antibody. These rats and 5 additional non-passively immunized rats were actively immunized with 1.0 ml of 0.25 per cent sheep erythrocytes 24 hours after passive immunization, and at 6-day intervals thereafter for a total

of 4 injections. The rats were bled for titers 6 days after each active immunization, the bleedings being done just before the second, third, and fourth active immunizations.

The 3 antisera were equally effective in markedly suppressing the primary hemolysin response. Furthermore, sustained suppression occurred in all rats in each passively immunized group. In contrast, the non-passively immunized rats had high titers at all bleedings. The experiment showed that 19S, 7S, or a mixture of 19S and 7S antibodies, in the doses given, were equally effective in suppressing the primary response, and in producing sustained suppression when injections of antigen were continued. The experiments were not adequate to exclude the possibility that suppression may have been due to a different antibody fraction than was tested for. Only the pooled primary response anti-sheep erythrocyte serum, containing predominantly 19S antibody, was used in subsequent experiments.

Inhibition of the Splenic Cellular Response by Passive Immunization.—The spleen is essential for hemolysin formation after intravenous injection of "low" doses of sheep erythrocytes into rats (8). Sheep erythrocytes and various other particulate antigens given *via* this route cause proliferation of pyroninophilic cells in spleens (7). X-Irradiation and certain cytotoxic agents, which depress antibody formation, inhibit this cellular proliferation (7). It was of interest to determine whether the apparently innocuous procedure of passive immunization with homologous antiserum would similarly inhibit splenic cellular proliferation associated with antigenic stimulation.

In a series of experiments, rats were passively immunized with 1.0 ml of anti-sheep erythrocyte serum and 24 hours later were injected with 1.0 ml of 2 or 4 per cent sheep erythrocytes. Control rats received antiserum only, sheep erythrocytes only, or no injections. The rats were sacrificed 4 or 6 days later. Passive immunization markedly suppressed the antibody response to these doses of antigen and also suppressed the increase in spleen weight and the hyperplasia of pyroninophilic cells. Passive immunization alone produced no apparent change in the spleen.

Inhibition of the Antibody Response by Passive Immunization after Active Immunization.—It was previously shown that passive immunization after active immunization inhibited hemolysin formation (1), and this observation was confirmed. Groups of 5 rats were passsively immunized with 1.0 ml of anti-sheep erythrocyte serum 24 hours before or 24 hours after active immunization with 1.0 ml of 0.25 per cent sheep erythrocytes. A third group of 5 rats was actively immunized without passive immunization. The mean 6 day titers were 0.4 (range 0 to 1) for rats passively immunized before active immunization, and 0.8 (range 0 to 1) for rats passively immunized after active immunization. The mean titer for the non-passively immunized rats was 5 (range 4 to 6).

EXPERIMENTAL OBSERVATIONS

Certain parameters of the phenomenon of suppression of the primary immune response by passive immunization were explored in the preliminary experiments. Passive immunization suppressed the cellular proliferation associated with antibody production, and this suggested that passive immunization inhibited antibody formation rather than antibody release from specifically stimulated cells. Passively given antibody and antigen may have formed complexes which altered the antigenicity of the antigen or altered its distribution. However, small doses of particulate antigen injected intravenously are cleared from the circulation rapidly, and passive immunization as late as 24 hours after active immunization was effective in suppressing the immune response. Therefore, the formation of complexes between passively given antibody and intact

circulating antigen was presumably not necessary for suppression of the antibody response.

A secondary hemolysin response was demonstrated in rats 3 to 4 weeks after primary immunization, when the titers of circulating hemolysin were low. As might be expected, passive immunization of previously immunized rats did not suppress the secondary hemolysin response (1). Apparently, the previously immunized animal had an altered capacity to respond to antigen in the presence of circulating antibody.

Experiments using passive immunization of intact animals gave no information concerning the mechanism of suppression of the primary response. Experiments were undertaken to determine whether circulating or cell-bound antibody produced the inhibition of antibody formation. The differential effect of passive immunization on the primary and secondary responses was explored.

Primary and Secondary Antibody Response to Sheep Erythrocyte–Antibody Complexes.—

Sheep erythrocyte-antibody complexes were produced *in vitro*. In one experiment 4.0 ml of anti-sheep erythrocyte serum inactivated at 56°C for 30 minutes was added to 2.0 ml of 5 per cent sheep erythrocytes. (Four ml of the antiserum was estimated sufficient for complete hemolysis of about 400 times the number of sheep erythrocytes in 2.0 ml of 5 per cent sheep erythrocytes in the presence of sufficient complement.) The suspension was incubated at 37°C for 15 minutes; hemolysis was absent but agglutination of erythrocytes occurred. The cells were washed 3 times in large volumes of normal saline, and after each washing the cells were mechanically dispersed by repeatedly passing the suspension through a fine needle. After the last washing the cells were resuspended in a volume of 20 ml of saline to give the equivalent of a 0.5 per cent suspension of sheep erythrocytes. Microscopically it could be seen that the suspension contained predominantly singly dispersed cells and only rare small aggregates of erythrocytes. The supernatant fluid of an aliquot of the washed suspension contained no measurable antibody. Although complexes were produced in the antibody excess, free antibody was removed. A second suspension of erythrocytes was prepared in an identical manner except that the cells were incubated with inactivated normal rat serum.

The antigenicity of the 2 suspensions was compared in normal rats and in rats immunized 4 weeks previously with 1.0 ml of 0.25 per cent sheep erythrocytes. The previously immunized rats had titers of 2 or less at the time of reimmunization. Five normal and 5 previously immunized rats were given 1.0 ml injections of 0.5 per cent suspension of erythrocytes incubated with immune serum and 5 normal and 5 previously immunized rats were injected with 1.0 ml of 0.5 per cent suspension of erythrocytes incubated with normal serum.

The results of the 6 day titers, presented in Table I, show that the erythrocyte-antibody complexes failed to elicit a measurable response in normal rats but produced a high secondary response in the previously immunized rats.

Another experiment was of similar design except that erythrocytes were incubated with antiserum which was not inactivated. The erythrocytes were completely hemolyzed; the stromata were washed and resuspended as in the

previous experiment. The erythrocyte stroma–antibody complexes failed to elicit an antibody response in normal rats but produced a high secondary response in previously immunized rats.

The results suggested that the suppression of antibody formation produced by passive immunization prior to active immunization might result from formation of antigen-antibody complexes in the circulation. Apparently such complexes formed in the circulation would not stimulate a primary response but would elicit a secondary response. The following experiments were designed to determine if antibody bound to spleen cells of non-immunized rats might also be effective in suppressing the primary antibody response of these cells.

TABLE I

Primary and Secondary Antibody Response to Sheep Erythrocyte–Antibody Complexes

	Immunization*	6-day antibody titers‡
Primary response§	Sheep erythrocytes	6.2 ± 0.80
Primary response§	Sheep erythrocyte–antibody complexes	0 ± 0
Secondary response‖	Sheep erythrocytes	7 ± 0.55
Secondary response‖	Sheep erythrocyte–antibody complexes	5 ± 0.55

* 1.0 ml of 0.5 per cent suspension of sheep erythrocytes injected intravenously. The erythrocytes, after incubation with normal rat serum or with anti–sheep erythrocyte serum, were washed and resuspended in saline.

‡ Mean titers for groups of 5 rats ± the standard error of the mean.

§ Normal rats.

‖ Rats immunized 4 weeks previously with 1.0 ml of 0.25 per cent sheep erythrocytes.

The Effect of Passive Immunization on the Primary Antibody Response of Normal Spleen Cells Transferred to X-Irradiated Rats.—A series of experiments was designed to measure the effect of passive immunization on the primary response of normal spleen cells transferred to rats made immunologically unresponsive by x-irradiation.

In different experiments spleen cells were "passively immunized": (*a*) after the spleen cells were injected into recipient rats, (*b*) *in vitro*, or (*c*) in the donor rats. The x-irradiated recipient rats were injected with spleen cells 3 to 4 hours after x-irradiation and were actively immunized 24 hours later. The rats were bled for antibody titers 6 days after active immunization. Each experiment included 3 control groups of 5 rats: x-irradiated rats not injected with spleen cells but actively immunized with sheep erythrocytes; non-irradiated rats passively immunized with anti–sheep erythrocyte serum before active immunization; and non-irradiated rats actively immunized with sheep erythrocytes. The 6 day antibody titers for these 3 control groups invariably showed: no measurable antibody titer for the x-irradiated rats; no, or very low, titers for the passively immunized non-irradiated rats; and high titers for the non-irradiated rats. Various other control groups were usually included.

The following experiment demonstrated that the phenomenon of suppression

of the hemolysin response could be reproduced with the spleen cell transfer system.

Ten x-irradiated rats were injected with 5×10^8 nucleated spleen cells obtained from normal rats. On the following day 5 of the rats were injected with 1.0 ml of the anti-sheep erythrocyte serum, and 2 hours later all rats were actively immunized with 1.0 ml of 0.5 per cent suspension of sheep erythrocytes.

TABLE II

The Effect of Passive Immunization on the Primary Antibody Response of Normal Spleen Cells Transferred to X-Irradiated Rats

	Spleen cells transferred to x-irradiated rats*	6-day antibody titer‡
Experiment 1		
Passive immunization of spleen cells in recipient rats	Normal cells§	3.2 ± 0.58
	Passively immunized cells§	0 ± 0
Experiment 2		
Passive immunization of spleen cells *in vitro*	Normal cells‖	3.4 ± 0.68
	Passively immunized cells‖	0.6 ± 0.60
Experiment 3		
Passive immunization of spleen cells in donor rats	Normal cells¶	3.6 ± 0.93
	Passively immunized cells¶	1.0 ± 0.63

* Recipient rats received 600 r total body irradiation and were actively immunized with 1.0 ml of 0.5 per cent suspension of sheep erythrocytes 24 hours after injection of spleen cells.

‡ Mean titers for groups of 5 rats ± the standard error of the mean.

§ 5×10^8 nucleated spleen cells transferred to each x-irradiated rat.

‖ 1.6×10^8 nucleated spleen cells transferred to each x-irradiated rat.

¶ 1.0×10^8 nucleated spleen cells transferred to each x-irradiated rat.

The results, recorded as Experiment 1, Table II, show that x-irradiated rats receiving normal spleen cells had moderate 6 day titers, and that passive immunization inhibited this response. The experiment did not indicate whether circulating or cell-bound antibody was responsible for the inhibition. The following experiments were designed to give information on this question.

Washed spleen cells pooled from normal donor rats were divided into two equal 5.0 ml samples; 5.0 ml of rat anti-sheep erythrocyte serum was added to one sample, and 5.0 ml of normal rat serum was added to the other sample. After incubation with the antiserum for 15 minutes at room temperature each cell suspension was centrifuged and washed twice in large volumes of medium. After the second washing the volume of each suspension was adjusted to contain 1.6×10^8 cells per ml. Five x-irradiated and 5 non-irradiated rats were injected with 1.0 ml of the cell suspension which had been incubated with the anti-sheep erythrocyte serum; similarly, 5 x-irradiated and 5 non-irradiated rats were injected with 1.0 ml of the other cell suspension. The following day all rats were actively immunized with 1.0 ml of a 0.5 per cent suspension of sheep erythrocytes.

The essential results, recorded as Experiment 2, Table II, showed that recipients receiving spleen cells incubated with the normal serum all responded with moderate antibody titers ranging from 1 to 4. In contrast, the recipients of spleen cells incubated with anti–sheep erythrocyte serum had no or very low titers. Control groups included non-irradiated rats which received the spleen cell suspension and were actively immunized; these rats had hemolysin titers equal to the titers of actively immunized non-irradiated controls, showing that the spleen cells "passively immunized" *in vitro* and then washed did not carry sufficient antibody to affect the primary antibody response of normal rats. Also, the supernatant fluid of an aliquot of the washed, "passively immunized" cell suspension contained no measurable antibody.

The essential findings of this experiment were confirmed in other experiments in which the donor rats were passively immunized with 1.0 ml of anti–sheep erythrocyte serum 24 hours prior to preparation of the spleen cells.

In one experiment two spleen cell suspensions were prepared, one from normal donor rats and one from rats passively immunized with anti–sheep erythrocyte serum. After washing the spleen cells, the volumes of the 2 suspensions were adjusted so that each suspension contained 1×10^8 nucleated spleen cells per ml. Ten x-irradiated rats each received 1.0 ml of the spleen cell suspension prepared from the normal rats, and 10 x-irradiated rats received 1.0 ml of the spleen cell suspension prepared for the passively immunized donor rats. The following day 5 x-irradiated rats in each group were actively immunized with 1.0 ml of 0.5 per cent sheep erythrocytes, and 5 x-irradiated rats in each group were actively immunized with 10 μg of flagella.

The results of the 6 day titers, recorded as Experiment 3, Table II, showed that x-irradiated rats which received spleen cells from normal rats responded to sheep erythrocytes with moderately high titers while rats which received cells from passively immunized donor rats had no or very low titers. The control groups of x-irradiated rats which received one or the other spleen cell suspensions and were immunized with flagella responded with equal agglutinin titers to *S. typhosa*; the agglutinin titers to *S. typhosa* were 4.8 (range 3 to 6) for rats receiving normal spleen cells, and 4.0 (range 2 to 6) for rats receiving cells from animals passively immunized with anti–sheep erythrocyte serum. Thus, the 2 cell suspensions had an equal capacity to respond to an antigen other than sheep erythrocytes.

These experiments demonstrated that specific antibody, undoubtedly bound to cells, effectively suppressed antibody formation. In Experiments 2 and 3, Table II, 24 hours elapsed between cell transfer and active immunization. This interval should have been sufficient for any exchange or equilibrium to occur between passively given antibody bound to spleen cells and the circulating proteins of the x-irradiated recipients. Thus, a small amount of antibody, apparently bound onto or into potential antibody-forming cells markedly suppressed the responsiveness of these cells to sheep erythrocytes.

In the following experiments the effect of passive immunization on spleen cells from previously immunized rats was measured.

The Effect of Passive Immunization on the Secondary Antibody Response of Spleen Cells Transferred to X-Irradiated Rats.—

In a typical experiment, spleen cells were obtained from donor rats that had been actively immunized with 1.0 ml of 0.25 per cent sheep erythrocytes 4 weeks previously; the donor rats had very low circulating titers at the time of sacrifice. 1×10^8 nucleated spleen cells ("immune spleen cells") were injected into each of 10 rats 4 hours after x-irradiation. The following day,

TABLE III

The Effect of Passive Immunization on the Secondary Antibody Response of Spleen Cells Transferred to X-Irradiated Rats

	Spleen cells from previously immunized donors*	Passive immunization‡	Active immunization§	6-day antibody titers
X-Irradiated rats¶	+	0	+	7.0 ± 1.08
	+	+	+	5.0 ± 0.55
	+	0	0	0 ± 0
	0	0	+	0 ± 0
Normal rats	0	0	+	4.8 ± 0.20
	0	+	+	0 ± 0

* Donor rats actively immunized with 1.0 ml of 0.25 per cent suspension of sheep erythrocytes 4 weeks before sacrifice.

‡ Each passively immunized rat injected with 1.0 ml of anti-sheep erythrocyte serum 8 hours before active immunization.

§ Each immunized rat injected with 1.0 ml of 0.5 per cent suspension of sheep erythrocytes.

‖ Mean titers for groups of 5 rats ± the standard error of the mean.

¶ Rats each received 600 r total body x-irradiation.

5 of the x-irradiated rats were passively immunized with 1.0 ml of the rat anti-sheep erythrocyte serum, and 8 hours later all 10 rats were actively immunized with 1.0 ml of 0.5 per cent sheep erythrocytes. X-Irradiated control rats included one group which received spleen cells but was not actively immunized, and one group which was actively immunized only. Non-x-irradiated control rats included one group of normal rats which was passively immunized before active immunization and one group which was actively immunized only.

The results, recorded in Table III, showed that the x-irradiated rats which received immune spleen cells and were actively immunized had a mean titer of 7; passive immunization reduced the mean titer to 5. The x-irradiated rats which received immune spleen cells alone or sheep cells alone had no measurable antibody response. The mean titer for the primary response of the non-irradiated normal controls was 5 and passive immunization abolished the primary response.

Two findings were striking: First, the antibody response of spleen cells from previously immunized rats was considerably higher than the antibody response produced by spleen cells from normal rats in the previous experiments. Secondly, passive immunization produced much less suppression of the secondary response. These findings were confirmed.

For example, in one experiment 2 suspensions of spleen cells were prepared under identical conditions; one suspension was prepared from rats actively immunized with 1.0 ml of 0.25 per cent sheep erythrocytes 5 weeks previously and the other cell suspension was prepared from normal rats. Five x-irradiated rats, each injected with 5×10^7 nucleated spleen cells from the previously immunized rats and then actively immunized with 1.0 ml of 0.5 per cent suspension of sheep erythrocytes, had a mean 6 day titer of 6 with a range of 5 to 7. Five x-irradiated recipients, each injected with tenfold more or 5×10^8 nucleated spleen cells from normal rats and actively immunized with the same dose of sheep erythrocytes, had a mean 6 day titer of 3 with a range of 1 to 4. The second finding was confirmed in 3 experiments which showed that passive immunization suppressed only slightly the secondary response of spleen cells from previously immunized rats transferred to x-irradiated rats.

Suppression of the Antibody Response by Primary Active Immunization with Small Doses of Antigen.—The above experiments showed that an initial antigenic stimulation in the absence of antibody apparently produced a population of cells capable of responding to antigen in the presence of antibody. However, antibody, circulating or bound to cells, suppressed the primary antibody response. Therefore, it seemed likely that after primary immunization, for as long as antibody was produced, subsequent response to injections of the same antigen might be limited to the population of cells originally specifically stimulated, or to the progeny of such cells. The production of antibody by these cells might suppress the response of potential antibody-forming cells which were not stimulated during the first immunization, or which matured after the first immunization. In this manner, primary immunization with a critical small dose of antigen, resulting in the formation of a small amount of antibody, might suppress antibody formation to subsequent injections of antigen.

An immunizing dose of 1.0 ml of 0.25 per cent sheep erythrocytes, containing about 5×10^7 erythrocytes, regularly produced a moderately high 6 day antibody response in normal rats. In a series of experiments groups of rats were injected intravenously with 1.0 ml of suspension containing 5×10^7 sheep erythrocytes or with 1.0 ml of various dilutions of the same suspension. Some rats receiving a dose of 1×10^7 sheep erythrocytes responded with as high titers as rats that received a dose of 5×10^7 sheep erythrocytes; other rats receiving 1×10^7 sheep erythrocytes responded with very low or no measurable titers. Invariably, rats which received 5×10^6 or fewer sheep erythrocytes responded with no measurable titers. Rats receiving the various doses of sheep erythrocytes were reinjected with 5×10^7 sheep erythrocytes 4 or 6 days after the primary immunization and were bled for titers 6 days after the second immunization. Rats initially injected with 5×10^7 or with very low doses of less than 1×10^5 sheep erythrocytes responded with high titers to the second injection of 5×10^7 sheep erythrocytes. Some rats receiving an initial injection of 5×10^6, 1×10^6, or 5×10^5 sheep erythro-

cytes responded with moderately high titers to the second injection of 5×10^7 sheep erythrocytes while other rats receiving the same initial immunizations showed marked suppression of the antibody response to the second injection of 5×10^7 sheep erythrocytes.

It was not possible to select a single initial immunizing dose of sheep erythrocytes which suppressed in all rats the response to a second injection of 5×10^7 sheep erythrocytes.

The following experiment demonstrated these findings and also showed that suppression was sustained in some rats following multiple injections of antigen.

Five rats were each injected with 1.0 ml of suspension containing 5×10^7 sheep erythrocytes; 5 rats were each injected with 1.0 ml of suspension containing 5×10^6 sheep erythrocytes, and 5 rats were each injected with 1.0 ml of a suspension containing 1×10^6 sheep erythrocytes. All 15 rats were further injected with 1.0 ml of a suspension containing 5×10^7 sheep erythrocytes, 6, 12, and 18 days after the first immunization. The rats were bled for titers just prior to each immunization and 6 days after the fourth immunization. The sera were stored and titrated at one time at the end of the experiment.

All rats receiving the initial immunization of 5×10^7 sheep erythrocytes had high titers during the course of the immunizations. None of the rats receiving the lower doses of antigen had measurable titers 6 days after the initial immunization. Five rats initially immunized with 5×10^6 or 1×10^6 sheep erythrocytes responded and maintained high or moderately high titers to the subsequent injections of the higher dose of 5×10^7 sheep erythrocytes. Four rats initially injected with the lower doses of antigen had no measurable antibody response to subsequent injections of 5×10^7 sheep erythrocytes; 1 rat initially injected with a lower dose of antigen responded to the fourth immunization. Titers for individual rats are recorded in Table IV. Thus, suppression was produced by an initial active immunization with a small dose of antigen, but suppression was not obtained as regularly as by passive immunization prior to the first active immunization.

Plaque-Forming Cells and Antibody Response Produced by Small Doses of Antigens.—Rats injected with small doses of 5×10^6 or fewer sheep erythrocytes had no measurable circulating antibody and showed a variable response to a second larger dose of sheep erythrocytes. A more sensitive method for measuring the immunological response to low doses of sheep erythrocytes might indicate the reason for this variability in the secondary response. The number of antibody-producing cells in spleens of mice injected with large numbers of sheep erythrocytes correlated with antibody titer (5, 6). Similar results were obtained in rats; the highest numbers of plaque-forming cells in spleens of rats injected with 5×10^7 or more sheep erythrocytes were found 4 to 6 days after immunization (9). This technique provided a possible means for determining the immunological response produced by low doses of antigen. Also, hemolytic antibody in low concentrations might be demonstrated on agar plates containing sheep erythrocytes.

Six rats were each injected with 1.0 ml of suspension containing 5×10^7 sheep erythrocytes; 6 rats were each injected with 1.0 ml of a suspension containing 5×10^6 sheep erythrocytes, and 6 rats were each injected with 1.0 ml of a suspension containing 5×10^5 sheep erythrocytes. Six uninjected rats served as controls. Spleens and blood were obtained from 3 rats in each group 4 and 6 days after immunization. Numbers of plaque-forming cells in spleens, and serum hemolysin titers demonstrated on agar plates, are recorded for individual rats in Table V.

TABLE IV

Suppression of the Antibody Response by Primary Active Immunization with Small Doses of Antigen

Rat No.	1st immunization with SRBC*	6 days, 2nd immunization with 5×10^7 SRBC	12 days, 3rd immunization with 5×10^7 SRBC	18 days, 4th immunization with 5×10^7 SRBC	24 days
		Antibody titer	Antibody titer	Antibody titer	Antibody titer
1	5×10^7	5	5	7	6
2	5×10^7	5	5	7	7
3	5×10^7	5	5	7	6
4	5×10^7	7	6	7	6
5	5×10^7	5	6	7	7
6	5×10^6	0	7	6	6
7	5×10^6	0	3	3	5
8	5×10^6	0	3	3	4
9	1×10^6	0	4	3	3
10	1×10^6	0	5	4	4
11	5×10^6	0	0	0	0
12	5×10^6	0	0	0	0
13	1×10^6	0	0	0	3
14	1×10^6	0	0	0	0
15	1×10^6	0	0	0	0

* Rats receiving 5×10^6 or 1×10^6 sheep erythrocytes on the first immunization were arbitrarily separated into 2 groups and numbered; one group showed no suppression and one group showed marked suppression of antibody titers to subsequent injections of 5×10^7 sheep erythrocytes.

The plaque-forming cell response varied considerably for rats receiving the same dose of antigen and sacrificed at the same interval after immunization. However, 5 to 238 plaque-forming cells per 10^6 recovered spleen cells were found for rats receiving the highest dose of antigen; sera from these rats contained antibody and titers for individual rats correlated with the number of plaque-forming cells obtained from their spleens. Rats injected with the 2 lower doses of antigen had increased numbers of plaque-forming cells in their spleens, although none had more than 3 plaque-forming cells per 10^6 recovered spleen cells. No antibody was demonstrated in any of the sera of the rats re-

TABLE V

Plaque-Forming Cells and Antibody Response Produced by Small Doses of Antigen

Rat No.*	Immunization‡	Days after antigen injection	Plaque-forming cells per spleen§	Plaque-forming cells per 10^6 spleen cells	Plate hemolysin titers	
					Whole serum	Highest serum dilution producing hemolysis
1	5×10^7 SRBC	4	93,000	111	4+	128
2			55,500	65	4+	64
3			3,800	5	1+	Whole
4		6	233,000	238	4+	512
5			148,000	214	4+	512
6			32,500	38	4+	128
7	5×10^6 SRBC	4	2,500	3	0	
8			1,000	1	0	
9			150	0.2	0	
10		6	2,700	3	0	
11			2,000	2	0	
12			400	0.6	0	
13	5×10^5 SRBC	4	1,500	2	0	
14			1,000	1	0	
15			350	0.5	0	
16		6	600	0.7	0	
17			450	0.6	0	
18			450	0.4	0	
19	None	4	83	0.2	0	
20			46	0.05	0	
21			38	0.04	0	
22		6	16	0.03	0	
23			28	0.03	0	
24			47	0.02	0	

* Rats arbitrarily numbered.

‡ Rats each injected intravenously with 1.0 ml of suspension containing 5×10^7, 5×10^6, or 5×10^5 sheep erythrocytes.

§ The total numbers of nucleated cells recovered from spleens varied from 4.9 to 10.2 $\times$ 10^8 cells per spleen.

ceiving the 2 lower doses of antigen. Individual rats, receiving either 5×10^6 or 5×10^5 sheep erythrocytes and sacrificed at the same time, had equivalent numbers of plaque-forming cells in their spleens. The experiment was adequate to demonstrate an immunological response to antigen in the absence of measur-

able serum antibody. The variable plaque-forming cell responses to the lower doses of antigen correlated with the variable suppression of the secondary response observed in the previous experiment.

DISCUSSION

It seems desirable to separate cells that are capable of responding to an antigen into 2 categories: "antibody-forming cells" and "potential antibody-forming cells." Some of the reasons for this separation have been based on reported differences between the primary and secondary responses. For example, the secondary response is less sensitive to inhibition by x-irradiation than the primary response (10–12). Also, the stimulation of antibody formation by addition of antigen to cells from previously immunized animals cultured *in vitro* (13, 14), or transferred to immunologically unresponsive recipients (15) is more easily achieved and with greater yield of antibody than when cells from normal animals are used. Recently it has been reported that normal cells are less sensitive to inhibition of antibody formation by actinomycin D than are cells from previously immunized animals (16).

The present studies emphasize another difference. Clearly, immunization produces cells capable of responding to antigen in the presence of antibody. Four findings supported this conclusion. (*a*) A secondary response was elicited when animals had circulating antibody at the time of reimmunization (1). (*b*) Passive immunization did not inhibit the secondary response (1). (*c*) Antigen-antibody complexes formed *in vitro* elicited a high secondary response. (*d*) Cells from previously immunized animals transferred to animals made unresponsive by x-irradiation responded to antigen in the presence of passively given antibody. In contrast, the antibody response of non-immunized animals or of cells from such animals was markedly inhibited by the presence of antibody. Three findings supported this conclusion. (*a*) The primary response was markedly inhibited by passive immunization. (*b*) Antigen-antibody complexes formed *in vitro* elicited no measurable primary antibody response. (*c*) Exposure of cells to antibody in non-immunized donors or *in vitro* suppressed their response to the antigen in x-irradiated recipients.

For the purposes of this discussion the designation of antibody-forming cells is applied to cells which have been modified by previous encounter with the antigen or possibly a closely related antigen. This designation does not exclude the possibility that some antibody-forming cells may be "genetically determined" in their capacity to respond to the antigen. Antibody-forming cells are considered to be cells which can respond to the antigen in the presence of specific antibody to the antigen. The designation potential antibody-forming cells is applied to cells from normal animals which at a given time have the capability of responding to a specific antigen. Potential antibody-forming cells are considered to be cells which are unresponsive to the antigen in the presence

of specific antibody to the antigen. The use of the terms, antibody-forming cells and potential antibody-forming cells, does not eliminate the possibility that more than one functional cell type may be included for one or the other designation.

It is generally accepted that antigen stimulates cell mitosis as an essential part of the early phase of antibody production. Hyperplasia of lymphoid tissue with increased numbers of pyroninophilic cells, indicating increased RNA synthesis, is a manifestation of this phase of the response. The inhibition of splenic hyperplasia by passive immunization provides indirect evidence that antibody prevents the initial stimulus of antigen to cause mitosis rather than preventing release of antibody from stimulated cells. It is of interest that passive immunization also suppresses the marked increase in numbers of plaque-forming cells in spleens of immunized rats (9), thus adding further support to the findings reported here.

Although formation of antigen-antibody complexes in the circulation may prevent stimulation of potential antibody-forming cells by the antigen, two findings strongly suggest that antibody adsorbed onto or into potential antibody-forming cells suppresses their response to antigen. Normal spleen cells exposed to antibody in donor rats or *in vitro* and then washed free of excess antibody had a suppressed response to the antigen. Active immunization with small doses of antigen which produced no detectable circulating antibody produced marked suppression of the secondary antibody response in some rats. The present experiments provide no information about the mechanism whereby antibody prevents the stimulation of potential antibody-forming cells by antigen. Presumably, the site of inhibition could be either on the cell surface or at some site in the internal structure of potential antibody-forming cells.

The variable suppression of the secondary response produced by initial immunization with small doses of antigen correlated with the variable numbers of plaque-forming cells found in the spleens. Sampling of only the spleen for plaque-forming cells was probably justified since splenectomy markedly decreased the antibody response of rats given small doses of sheep erythrocytes intravenously (8). Also, in other experiments plaque-forming cells were not found in lymph nodes of intact rats injected intravenously with small doses of sheep erythrocytes although a few plaque-forming cells were found in lymph nodes of rats injected intravenously with the very large dose of 10^{10} sheep erythrocytes (9). The failure to demonstrate circulating antibody in the presence of increased numbers of plaque-forming cells in rats receiving low doses of antigen could be due either to the insensitivity of the system for measuring antibody or to uptake of antibody by potential antibody-forming cells and possibly other cells. If the latter were the case, appreciable levels of circulating antibody would not result until the cells capable of taking up antibody were saturated.

The failure of a primary immunization to suppress the secondary response to a larger dose of antigen could occur in rats which had either a maximal or a minimal response to the small dose of antigen. Suppression presumably occurred when the initial antigenic stimulation was just sufficient to cause a balance between the number of antibody-forming cells and the amount of antibody produced. Sufficient antibody had to be produced to block potential antibody-forming cells from responding to the second injection of antigen; on the other hand, the numbers of antibody-forming cells resulting from the initial immunization could not be so numerous as to produce appreciable circulating antibody to the second injection of antigen.

In our experiments normal rats were unresponsive to antigen-antibody complexes formed *in vitro*; however, other antigen-antibody complexes may stimulate a primary response (17). The capacity of complexes to stimulate a primary response might be due to adjuvant-like activity of the antigen of the complexes, or because of dissociation of the complexes. On the other hand, an apparent primary response to antigen-antibody complexes could occur in animals in which there was some critical, but relatively small number of antibody-forming cells to the antigen (or a closely related antigen) of the complexes.

It has recently been reported that x-irradiated mice injected with normal mouse lymphoid cells incubated *in vitro* with mouse anti-*Salmonella adelaide* serum and then immunized with the *S. adelaide* antigen responded with as high titers as mice receiving untreated lymphoid cells (18). The reasons for difference between these findings and our findings are not known. Possibly the *Salmonella* antigen contained endotoxin or other components which have an adjuvant-like action that sheep erythrocytes do not have.

Immunological unresponsiveness has been produced in adult animals by various means. The suppression of the primary antibody response by passive immunization has been repeatedly observed in various species of animals and with various bacterial, viral, and cellular antigens, suggesting that the phenomenon is widespread in nature. These findings and the possible relationship between this phenomenon and immunological enhancement of tumor or skin homografts have been discussed (1, 18, 19). Both delayed hypersensitivity and circulating antibody production were suppressed in mature guinea pigs by feeding of a hapten (20) or by injecting very small amounts of the hapten or a soluble protein into mesenteric veins (21, 22). Unresponsiveness to heterologous serum proteins was induced in adult mice by initial injections of small quantities of soluble protein cleared of particulate matter having adjuvant-like activity. Partial unresponsiveness developed in some mice within 3 days after the initial injection of protein, and unresponsiveness developed regularly when the interval was 5 to 12 days between injections (23). Similar findings were reported using a comparable experimental design (24).

The fact that adult animals of different species can be made unresponsive to various antigens by either passive immunization or exposure of the animals

to small amounts of antigen would seem to indicate a common underlying principle. Our experiments suggest that antibody, either passively administered or actively produced in small quantities, may limit production of specific antibody to that portion of the antibody-forming system previously stimulated by the antigen. Thus, low levels of antibody formation may be responsible for maintaining immunological unresponsiveness. This suggestion is strengthened by the demonstration of antibody-forming cells in the absence of detectable circulating antibody. This mechanism may account in part for immunological unresponsiveness produced in certain other related experimental systems.

SUMMARY

Passive immunization of rats with homologous anti-sheep erythrocyte serum markedly inhibited the primary antibody response to various doses of sheep erythrocytes. Inhibition was "specific" and apparently produced by either "19S" or "7S" antibody to the antigen. Passive immunization inhibited splenic hyperplasia associated with the primary antibody response. Passive immunization 24 hours after active immunization effectively inhibited the primary antibody response.

The markedly suppressive effect of specific antibody on the primary antibody response contrasted sharply with the absence of this effect on the secondary response. Antigen-antibody complexes formed *in vitro* elicited no measurable primary antibody response but did elicit a high secondary response. Exposure of normal spleen cells to the antibody *in vivo* or *in vitro* suppressed their response to the antigen in x-irradiated recipients. In contrast, cells from previously immunized animals transferred to x-irradiated animals produced antibody in the presence of passively given antibody. Thus, "potential antibody-forming cells" from normal animals were unresponsive to the antigen in the presence of specific antibody, while "antibody-forming cells" from previously immunized animals responded to the antigen in the presence of antibody. Presumably, antibody actively produced in small quantities by a few antibody-forming cells might inhibit antibody formation by potential antibody-forming cells. Confirmation of this suggestion was obtained by showing that some animals initially injected with small doses of antigen failed to produce measurable antibody to subsequent injections of larger doses of the antigen. Low doses of antigen capable of inducing unresponsiveness produced no measurable circulating antibody, but these doses did produce increased numbers of plaque-forming (antibody-releasing) cells in spleens of rats. Thus, the formation of specific antibody may provide a homeostatic or "feed-back" mechanism which controls or limits production of specific antibody to the portion of the antibody-forming system previously stimulated by the antigen. This mechanism may account in part for immunological unresponsiveness produced in certain other related experimental systems.

BIBLIOGRAPHY

1. Neiders, M. E., Rowley, D. A., and Fitch, F. W., The sustained suppression of hemolysin response in passively immunized rats, *J. Immunol.*, 1962, **88,** 718.
2. Winebright, J., and Fitch, F. W., Antibody formation in the rat. I. Agglutinin response to particulate flagella from *Salmonella typhosa*, *J. Immunol.*, 1962, **89,** 891.
3. Uhr, J. W., and Finkelstein, M. S., Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage $\phi\chi$ 174, *J. Exp. Med.*, 1963, **117,** 457.
4. Kern, M., and Eisen, H. N., The effect of antigenic stimulation on incorporation of phosphate and methionine into proteins of isolated lymph node cells, *J. Exp. Med.*, 1959, **110,** 207.
5. Jerne, N. K., and Nordin, A. A., Plaque formation in agar by single antibody-producing cells, *Science*, 1963, **140,** 405.
6. Jerne, N. K., Nordin, A. A., and Henry, C., The agar plaque technique for recognizing antibody-producing cells, *in* Cell-bound Antibodies, (B. Amos and H. Koprowski, editors), Philadelphia, The Wistar Institute Press, 1963, 109.
7. Wissler, R. W., Fitch, F. W., LaVia, M. F., and Gunderson, C. H., The cellular basis for antibody formation, *J. Cell. and Comp. Physiol.*, 1957, **50,** suppl. 1, 265.
8. Rowley, D. A., The effect of splenectomy on the formation of circulating antibody in the adult male albino rat, *J. Immunol.*, 1950, **64,** 289.
9. Fitch, F. W., and Rowley, D. A., unpublished data.
10. Dixon, F. J., Talmage, D. W., and Maurer, P. H., Radiosensitive and radioresistant phases in the antibody response, *J. Immunol.*, 1952, **68,** 693.
11. Talmage, D. W., Freter, G. G., and Thompson, A., The effect of whole body X-radiation on the specific anamnestic response in the rabbit, *J. Infect. Dis.*, 1956, **99,** 246.
12. Stoner, R. D., and Hale, W. M., Radiation effects on primary and secondary antibody responses, *in* The Effects of Ionizing Radiations on Immune Processes, (C. A. Leone, editor), New York, Gordon and Breach Science Publishers, 1962, 183.
13. Stavitsky, A. B., *In vitro* studies of the antibody response, *Advances Immunol.*, 1961, **1,** 211.
14. Michaelides, M. C., and Coons, A. H., Studies on antibody production. V. The secondary response *in vitro*, *J. Exp. Med.*, 1963, **117,** 1035.
15. Cochrane, C. G., and Dixon, F. J., Antibody production by transferred cells, *Advances Immunol.*, 1962, **2,** 205.
16. Weiler, E., Immunologically determined and competent cells are affected differentially by actinomycin D, *Science*, 1964, **144,** 846.
17. Uhr, J. W., Immunization with antigen-antibody complexes, *in* Mechanism of Antibody Formation, (M. Holub and L. Jarošková, editors), Prague, Publishing House of the Czechoslovak Academy of Sciences, 1960, 157.
18. Möller, G., Antibody-induced depression of the immune response: a study of the mechanism in various immunological systems, *Transplantation*, 1964, **2,** 405.
19. Uhr, J. W., and Bauman, J. B., Antibody formation. I. The suppression of anti-

body formation by passively administered antibody, *J. Exp. Med.*, 1961, **113,** 935.

20. Chase, M. W., Inhibition of experimental drug allergy by prior feeding of the sensitizing agent, *Proc. Soc. Exp. Biol. and Med.*, 1946, **61,** 257.
21. Battisto, J. R., and Miller, J., Immunological unresponsiveness produced in adult guinea pigs by parenteral introduction of minute quantities of hapten or protein antigen, *Proc. Soc. Exp. Biol. and Med.*, 1962, **111,** 111.
22. Battisto, J. R., and Chase, M. W., Immunological unresponsiveness to sensitization with simple chemical compounds, *J. Exp. Med.*, 1963, **118,** 1021.
23. Dresser, D. W., Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen, *Immunology*, 1962, **5,** 378.
24. Dietrich, F. M., and Weigle, W. O., Immunologic unresponsiveness to heterologous serum proteins induced in adult mice and transfer of the unresponsive state, *J. Immunol.*, 1964, **92,** 167.