THE MECHANISM OF TOLERANCE PRODUCED IN RATS TO SHEEP ERYTHROCYTES

II. The Plaque-Forming Cell and Antibody Response to Multiple Injections of Antigen Begun at Birth*

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Rats 4 weeks or older receiving a single injection of sheep erythrocytes had large numbers of plaque-forming cells in their spleens and high titers of 19S antibody. Repeated injections of the antigen given at 3- or 4-day intervals resulted in a marked decrease in numbers of plaque-forming cells in the spleens, although titers of 19S and 7S antibody remained elevated. The numbers of plaque-forming cells and antibody titers remained approximately constant as injections of antigen were continued. This stabilization of numbers of plaque-forming cells was associated with the short interval between antigen injections, since recovery of the response occurred when the interval between antigen injections was lengthened. Thus, the relatively constant exposure of the animals to an antigen provided a mechanism for controlling or limiting the response of antibody-forming cells to that antigen (1).

Previous studies suggested another mechanism for controlling or limiting the antibody response to an antigen (2). Specific antibody absorbed onto or incorporated into "potential antibody-forming cells" from normal animals prevented these cells from responding to the antigen. Antibody did not have this effect on "antibody-forming cells" from animals previously immunized with that antigen. Thus, the formation of specific antibody provided a homeostatic or feed back mechanism for limiting antibody production to that portion of the antibody-forming system previously stimulated by the antigen.

The data to be reported support the concept that these two mechanisms, one the result of relatively constant antigenic stimulation, and the other the result of formation of small amounts of antibody, are responsible for the induction and maintenance of tolerance to sheep erythrocytes produced in rats.

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Materials and Methods

The rats and antigen injections were previously described in detail (1). The antigen was 0.2 ml of 20 per cent suspension of washed whole erythrocytes injected intraperitoneally. Rats receiving antigen injections beginning at the day of birth are referred to as "tolerant" animals for convenience. Tolerant rats were always injected on the day of birth and twice per week thereafter at regularly alternating 3- and 4-day intervals. The plaque-forming cell and antibody response of tolerant rats was compared with 2 control groups of the same age and sex: 1 group received a single injection of antigen at the time when the tolerant rats received the last injection of antigen; the 2nd group was non-immunized.

Plaque-Forming Cells.—The modified technique of Jerne and Nordin for demonstrating plaque-forming cells in spleens has been described (1, 2). The preparation and counting of plates for animals receiving a single antigen injection were as previously described (1). Since spleens of non-immunized and tolerant rats contained relatively few plaque-forming cells, plates were inoculated with larger numbers of spleen cells and additional precautions were taken in counting plates prepared from these animals. Spleens of all tolerant and non-immunized animals were prepared to give a final volume of 2.5 ml in the tissue culture medium. Two-tenths ml of the spleen cell suspension (about 4×10^7 nucleated spleen cells) were inoculated in each plate; 5 plates were prepared for each tolerant and each non-immunized animal. After 1 hour incubation and before the addition of complement, all plates were examined using a magnification of 10 to 25. Any defect in the spleen cell–sheep erythrocyte layer which could be mistaken for a plaque was marked by ink on the underside of the plate and was not counted subsequently. Complement was added to 3 plates from each animal; the same volume of the same complement preparation, inactivated at 56°C for 30 minutes, was added to the remaining 2 plates from each animal. The plates were incubated for 1 hour with complement, washed with saline, and counted immediately. Very few plaques developed on plates incubated with inactivated complement (a total of 9 "plaques" was found for 3 non-immunized and 4 tolerant rats; no plaques were present in the plates from other non-immunized and tolerant rats incubated with inactivated complement). No correction was made for the plaques counted on the plates incubated with inactivated complement. The plaques on plates incubated with active complement were circled by a sharp needle; each plaque was inspected with a conventional microscope at a magnification of 40 and 100 when plaques were not close to the edge of the plate. Occasional plaques contained aggregates of tissue debris or possibly extraneous material, and were not counted.[1]

The number of nucleated cells recovered from spleens was remarkably constant. Within experiments, differences in recovery never varied more than 10 per cent between groups of tolerant and non-immunized rats, and rats which received a single injection of antigen. Differences in recovery of cells from these 3 groups were not consistent. The data are expressed as numbers of plaque-forming cells per total spleen rather than as per number of recovered spleen cells, because relatively few plaque-forming cells were present in spleens of tolerant rats.

Antibody Titers.—The titration of sera for hemagglutinins has been described in detail (1). Titrations were for total antibody and for antibody present after treatment of sera with 2-mercaptoethanol (2-ME), designated for convenience as 7S antibody. The titrations used the double dilution technique with an initial serum dilution of 1:10. Titers were recorded

[1] Various bacterial contaminants may produce "plaques" which are made more apparent by active complement. These plaques have been observed to appear 3 to 6 hours after incubation with complement; *i.e.* 5 to 8 hours after spleen cells are harvested and plated. This possible source of experimental error is decreased or eliminated by the short incubation period of 1 hour without complement and 1 hour with complement, rinsing the plates and counting them immediately.

as the number of the last tube showing 1 + hemagglutination; thus, the titer expressed the serum dilution of 10×2^n where n is the tube number. Mean titers for groups of rats $\pm$ the standard error of the mean are reported for Table II. A titer of 0 was arbitrarily assigned to sera showing less than the end point reaction at a serum dilution of 1:10.

Plate Hemolysin Titers.—Agar plates were prepared as for demonstrating plaque-forming cells (2), but only sheep erythrocytes were added to agar in the thin overlayer. Small droplets of heat-inactivated serum and of serial twofold dilutions of the serum were spotted on the plate. The plates were incubated briefly, rinsed with saline and flooded with fresh rat complement, and incubated again. Serum-containing antibody produced clear plaques of complete hemolysis 2 to 3 mm in diameter. Higher dilutions of antibody produced distinct plaques of partial hemolysis.

EXPERIMENTAL OBSERVATIONS

The Plaque-Forming Cell and Antibody Response of Tolerant Rats.—The maximum interval between injections of antigen was 4 days. It was, therefore, of interest to measure the plaque-forming cell and antibody response at this interval in rats which had received various numbers of antigen injections. Furthermore, the response 4 days after the last antigen injection could be compared with the response of the adult rats immunized in the same manner (1).

New-born rats from 5 litters delivered on the same day were injected with antigen; injections were repeated twice a week at regularly alternating 3- and 4-day intervals. The response of the tolerant rats was compared with: (*a*) the response of rats of similar or equal age which received a single antigen injection when the tolerant rats received their last antigen injection; and (*b*) rats of the same age which received no immunization. Groups of 4 to 6 tolerant rats, along with groups of control rats, were sacrificed 4 days after they had received a total of 5 to 42 antigen injections. The responses of the individual rats are recorded in Table I.

All 6 rats receiving 5 antigen injections (injected at 1, 4, 7, 11, and 14 days of age and sacrificed at age 18 days) had 3000 to 22,000 plaque-forming cells in their spleens and antibody titers of 2 to 4. Their response was equivalent to the response of control rats which received a single antigen injection.

It was previously shown that rats receiving a single antigen injection on the day of birth or at age 7 days had no measurable plaque-forming cell and antibody response (1). Apparently, the first 3 or 4 antigen injections produced no appreciable response and the 5th antigen injection produced a response equivalent to that of a single antigen injection given at this age. Clearly, these rats which received only 5 antigen injections were not immunologically unresponsive or tolerant.

The responses for 16 rats, sacrificed 4 days after they had received 15, 17, 22, or 30 antigen injections, contrasted sharply with the responses of the rats which received only 5 antigen injections. The 16 rats had from 143 to 1800 (mean 504) plaque-forming cells in their spleens, about 8 times as many plaque-forming cells per spleen as the non-immunized controls. The hemagglutinin titers were 0 for

TABLE I

Plaque-Forming Cell and Antibody Response to Multiple Antigen Injections Begun at Birth

No. of Injections	Tolerant‡		Controls		
			Non-injected§	Single injection‡	
	Plaque-forming cells per spleen	Antibody titer	Plaque-forming cells per spleen	Plaque-forming cells per spleen	Antibody titer
5*	22,000	4	144	29,000	8
	21,000	4	113	18,000	7
	7,000	4	113	10,000	7
	6,000	3		8,000	8
	6,000	2		5,000	7
	3,000	4			
15	268	0	68	946,000	6
	208	0	43	826,000	5
	158	0	38	358,000	4
	143	0	8		
17	1,800	0	100	386,000	6
	750	2	50	436,000	7
	483	0	42	275,000	6
	275	0	42		
22	740	3	125	87,000	7
	583	3	83	40,000	8
	493	2	58	28,000	7
	283	0	40	27,000	8
30	788	0	131	466,000	7
	569	2	106	414,000	8
	288	2	75	228,000	6
	238	0	63	125,000	6
			50	48,000	5
42	563	8	100	610,000	8
	438	3	88	190,000	7
	325	1	75	183,000	6
	250	7	63	97,000	7
			63	97,000	6
			38	75,000	7

* The tolerant and non-injected controls were 18 days old at sacrifice; the single injection controls were 21 days old at sacrifice. The tolerant rats receiving more than 5 antigen injections and their controls were the same age at sacrifice.

‡ All tolerant rats were sacrificed 4 days after the last antigen injection; all control rats were sacrificed 4 days after the single antigen injection. Results for animals within groups have been arbitrarily arranged according to numbers of plaque-forming cells in spleens.

§ Antibody titers were 0 for all non-injected controls.

10 of the tolerant rats, and 2 to 3 for 6 of the tolerant rats. The numbers of plaque-forming cells in spleens of the tolerant rats did not correlate with hemagglutinin titers. Four tolerant rats, age 146 days, sacrificed 4 days after the 42nd antigen injection, showed no increase in numbers of plaque-forming cells; but all 4 animals had hemagglutinin titers ranging from 1 to 8. The hemagglutinin titers were 0 for all the non-immunized control rats. The control rats, which received a single injection of antigen, had large numbers of plaque-forming cells in their spleens and antibody titers ranging from 4 to 8.

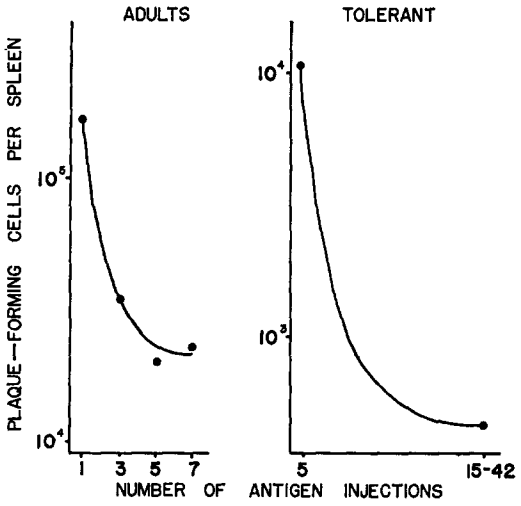

FIG. 1. Comparison of the decline of the plaque-forming cell response produced by multiple antigen injections in adult and tolerant rats. Antigen injections were given twice a week. The response was measured 4 days after the last antigen injection. The data for the adult rats were presented in detail previously (1).

The animals receiving 5 antigen injections had responses equivalent to rats of similar age which had received only a single antigen injection. The animals receiving 15 to 42 antigen injections had about $\frac{1}{20}$ the number of plaque-forming cells in their spleens as the animals receiving 5 antigen injections. The numbers of plaque-forming cells produced by more than 5 antigen injections stabilized as antigen injections were continued. Adult animals receiving 3 or more injections of the same dose of antigen as the tolerant rats in the present experiment had decreased numbers of plaque-forming cells in their spleens; the numbers of plaque-forming cells also stabilized with continued antigen injections (1). These findings for adult and tolerant rats are illustrated in Fig. 1.[2]

[2] In other experiments, tolerant rats were sacrificed 3 or 4 days after they had received 7, 9, or 13 antigen injections. The numbers of plaque-forming cells in the spleens of these animals were comparable to the numbers of plaque-forming cells recorded for the tolerant rats receiving 15 to 42 antigen injections in Table I. Thus, the sharp drop in numbers of plaque-forming cells plotted for the tolerant animals in Fig. 1 is probably justified.

Repeated antigen injections produced a greater decrease in the plaque-forming cell response in the tolerant rats than in rats immunized as adults. However, this difference was not great, and the time sequence for the decrease was similar for the adult and the tolerant rats. The findings suggested that the mechanism producing the decline in numbers of plaque-forming cells was similar in both groups of animals.

Tolerant rats which had received 13 or 22 antigen injections were sacrificed 15, 18, or 32 days after the final antigen injection. The numbers of plaque-forming cells in the spleens of these animals were always lower than in comparable tolerant rats sacrificed 4 days after the last antigen injection. The antibody titers were 0 for these rats. Thus, no late rise in numbers of plaque-forming cells

TABLE II

Recovery of the Plaque-Forming Cell and Antibody Response in Tolerant Rats

	Days between the last 2 antigen injections*			Controls	
	3 (4 rats)	14 (4 rats)	28 (5 rats)	Non-injected (5 rats)	1 antigen injection (5 rats)
Plaque-forming cells per spleen‡	470 ± 128	1,790 ± 480	4,410 ± 2,080	85 ± 15	256,000 ± 113,000
Antibody titer‡	1.0 ± 0.58	0	4.4 ± 0.24	0	6.4 ± 0.51

* The rats received a total of 23, 27, or 30 antigen injections.

‡ Animals sacrificed 4 days after the last antigen injection. Results are expressed as the mean ± the standard error of the mean.

or in antibody titers occurred with cessation of antigenic stimulation, a finding identical to that observed in rats similarly immunized as adults (1).

The following experiment was designed to determine whether an increased interval between the last 2 antigen injections of a series resulted in recovery of the plaque-forming cell and antibody response in tolerant rats.

Recovery of the Plaque-Forming Cell and Antibody Response in Tolerant Rats.— The spleens of tolerant rats which had received 15 antigen injections contained low numbers of plaque-forming cells; the numbers of plaque-forming cells stabilized at this low level as antigen injections were continued. Similar stabilization of plaque-forming cells, but at a higher level, occurred when rats received repeated antigen injections as adults. A progressive recovery of the plaque-forming cell response occurred in adults rats when the interval between the last 2 antigen injections was increased. It was of interest to determine whether similar recovery occurred in tolerant rats.

The tolerant rats were litter mates of the animals tested in the previous experiment, Table I. Antigen injections were discontinued for 1 group of tolerant rats after they had received 22 antigen injections, and for a 2nd group of tolerant rats after they had received 26 antigen injections. Antigen injections

were continued for a 3rd group of tolerant rats. All 3 groups were injected with antigen when the 3rd group received the 30th antigen injection. Thus, the interval between the 22nd and 23rd antigen injections was 4 weeks for 1 group, and the interval between the 26th and 27th antigen injections was 2 weeks for the 2nd group. The rats were sacrificed 4 days after the final antigen injection along with non-immunized animals and animals which had received a single antigen injection. The responses of these animals are presented in Table II. (The individual responses for the tolerant rats receiving 30 antigen injections and for the controls were presented in Table I.)

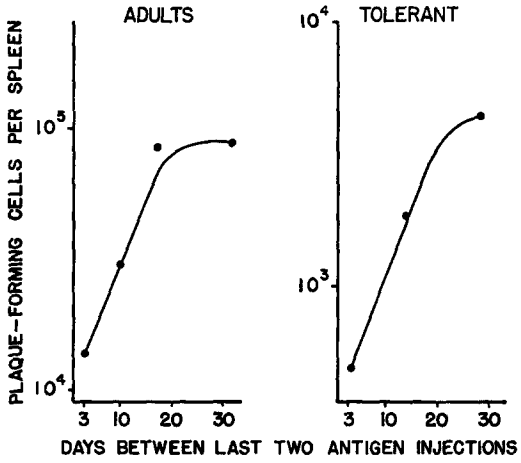

FIG. 2. Comparison of the recovery of the plaque-forming cell response produced by increasing the interval between the last 2 antigen injections in adult and tolerant rats. The response was measured 4 days after the last antigen injection. The data for the adult rats were presented in detail previously (1).

When the interval between the last 2 antigen injections was 2 weeks, the rats had about 4 times as many plaque-forming cells in their spleens as the tolerant rats receiving continuous antigen injections; the antibody titers were 0 for these rats. When the interval between the last 2 antigen injections was 4 weeks, the rats had about 9 times as many plaque-forming cells in their spleens as the tolerant rats receiving continuous antigen injections; all of these rats had moderately high antibody titers of 4 to 5 (titers for 7S antibody ranged from 1 to 3).

The recovery of the plaque-forming cell response of the tolerant rats is compared with the recovery in rats immunized as adults (1) in Fig. 2. An interval of 4 weeks between antigen injections resulted in a slightly greater recovery in the tolerant rats than in the rats immunized as adults. The time sequence for the recovery of the response was similar for the 2 groups. The findings sug-

gested that the mechanism producing recovery of the plaque-forming cell and antibody response was similar in both groups of animals.

The Antibody Response of Tolerant Rats.—The presence of circulating antibody in some tolerant rats provided evidence that the increased numbers of plaque-forming cells in tolerant rats was indicative of increased antibody formation. However, some rats had no detectable antibody by the hemagglutinin test. Plate hemolysin titrations, providing a more sensitive method for demonstrating circulating antibody in whole serum or low dilutions of serum, were done on sera from the tolerant and non-immunized rats reported in Table I. Sera from tolerant rats having hemaglutinin titers of 1 or higher all produced "plaques" of complete hemolysis at a serum dilution of 1:10 and were not titrated at higher serum dilutions. Sera from 9 of 10 tolerant rats having negative hemagglutinin titers (serum dilution of 1:10) produced plaques of complete to partial hemolysis at dilutions of 1:2 to 1:32. A single undiluted serum from a tolerant rat which received 17 antigen injections and had 483 plaque-forming cells in its spleen produced no hemolysis. Undiluted sera from several non-immunized rats produced a trace of hemolysis; none of the sera from non-immunized rats diluted 1:2 produced hemolysis.

The plate hemolysin technique was used to test sera for hemolytic antibody after treatment with 2-ME, but the lowest serum dilution which could be tested was 1:5. The sera from tolerant rats having hemagglutinin titers of 1 or higher contained 7S antibody in serum dilutions of 1:5; two sera from tolerant rats having negative hemagglutinin titers had 7S antibody in serum dilutions of 1:5. The findings were sufficient to indicate that some tolerant rats produced small amounts of a mixture of 19S and 7S antibody. Thus, the tolerant rats had more plaque-forming cells in their spleens than non-immunized rats, and all but 1 of the tolerant rats had demonstrable circulating antibody. The amount of circulating antibody did not correlate well with numbers of plaque-forming cells in the spleens of individual tolerant rats, but this absence of correlation was also observed for rats which received multiple antigen injections as adults (1).

The Inhibition of the Plaque-Forming Cell Response by Antibody.—Small quantities of anti-sheep erythrocyte antibody given passively or produced by active immunization inhibited the antibody response of potential antibody-forming cells (2, 3). The antibody produced by relatively few antibody-forming cells in tolerant rats would presumably prevent potential antibody-forming cells of tolerant rats from responding to the antigen. In the following experiment, passively-given antibody produced profound inhibition of the plaque-forming cell response of growing rats.

Each of six 16-day-old rats was injected intravenously with 0.5 ml of rat anti–sheep erythrocyte serum, a dose calculated to give circulating antibody titers of 1 to 2 at 24 hours after passive immunization. The following day these rats and 6 litter mates, which served as controls, were each injected intraperitoneally

with 0.2 ml of 20 per cent sheep erythrocytes. All were sacrificed 4 days later. The spleens of the passively immunized rats contained 25 to 200 plaque-forming cells per spleen; the spleens of the control rats contained 4000 to 18,000 plaque-forming cells per spleen.

Presumably sustained antibody production at low levels by the tolerant rats would inhibit potential antibody-forming cells from responding to the antigen.

DISCUSSION

Objections can be raised to use of the word "tolerance" to describe the type of immunological unresponsiveness we have studied, since this word has been used by many to describe the absence of homograft rejection and the latter is generally considered to be mediated by "hypersensitive cells" and not by circulating antibody. The suppression of antibody formation is not necessarily equivalent to suppression of hypersensitivity. Moreover, for some biologists, tolerance by definition means the complete absence of an immunological response. The tolerant animals in the present experiments had a demonstrable immune response to the antigen used to produce tolerance. For the present, we do not believe that a rigid definition of the word is desirable, since the same biological mechanisms may be involved in the induction and maintenance of immunological unresponsiveness produced by various experimental procedures.

Various procedures besides neonatal injections of antigen suppress both the circulating antibody response and hypersensitivity reactions. Both delayed hypersensitivity and circulating antibody can be suppressed in mature guinea pigs by feeding of a hapten or by injecting very small amounts of a hapten or a soluble protein into mesenteric veins (4–6). Passive immunization of adult animals may produce immunological enhancement of tumor or skin grafts (7, 8). Thymectomy, X-irradiation, chronic drainage of lymph from the thoracic duct, and other procedures producing involution, depletion, or destruction of lymphoid tissue may suppress both kinds of immunological responses (9–12).

Clearly, animals are tolerant if there is complete absence of an immunological response to an antigen, but we do not know of experimental systems where it has been shown conclusively that there was complete absence of an immunological response to the antigen in the tolerant animals. For example, homograft survival alone may not be valid evidence for the absence of an immune response since various procedures which do not abolish the immune response may result in prolonged homograft survival. Obviously, the sensitivity of the assay system will determine whether or not an immune response can be detected. In the present experiments, it required a method capable of detecting a few hundred antibody-forming cells in an entire spleen (using an antibody titration system sufficiently sensitive to detect antibody released from single cells) to demonstrate an immunologic response in some tolerant animals. Therefore, it seems reasonable to expect that extremely sensitive methods will be required to determine

whether or not an active immune process is essential for maintenance of tolerance to other antigens and to homografts.

The reasons for separating cells capable of responding to antigen into two categories, "potential antibody-forming cells" and "antibody-forming cells," were discussed in detail (2). The designation of *potential antibody-forming cells* is applied to cells from normal animals which at a given time are capable of responding to a specific antigen. However, these cells are unresponsive to antigen in the presence of specific antibody to the antigen. The designation of *antibody-forming cells* is applied to cells which have been modified by previous encounter with the antigen. These cells respond to the antigen in the presence of specific antibody to the antigen. These designations are used primarily for the sake of convenience in discussion; the terms may describe two completely different classes of cells or may merely indicate the extremes of a spectrum of functional states of a single class of cells. On the other hand, the category of potential antibody-forming cells may include two different cell types such as macrophages and lymphocytes, while the category of antibody-forming cells may constitute a class of cells which can respond to antigen directly.

Two mechanisms are proposed to explain the induction and maintenance of tolerance, one which controls or limits the response of antibody-forming cells, and the other which controls or limits the induction or transformation of potential antibody-forming cells to antibody-forming cells. The following evidence supports the operation of the first mechanism. Rats receiving repeated injections had stabilized numbers of plaque-forming cells in their spleens. When antigen injections were begun in adults, the numbers of antibody-forming cells stabilized at high levels, and these animals had high antibody titers. When injections were begun at birth, the numbers stabilized at low levels, and the animals had very low titers of antibody. Presumably, the mechanism producing stabilization of numbers of plaque-forming cells and antibody titers was the same whether antigen injections were begun in adults or at birth. An appreciable increase in plaque-forming cells and antibody response occurred when the interval between the last two of a series of antigen injections was increased. This was true although the antibody titer at the time of the last injection remained elevated. The rate of recovery was the same for tolerant rats and rats immunized as adults, suggesting that a similar process operated in both. It seems likely that repeated closely spaced antigen injections interfere with either division or maturation of antibody-forming cells. A stabilization of numbers of plaque-forming cells and antibody titer results; as the interval between antigen injections is increased, additional antibody-forming cells mature or are formed by cell division (1).

The mechanism producing stabilization of numbers of plaque-forming cells and antibody titers would not explain why stabilization occurred at low levels for tolerant rats and at high levels for rats injected as adults. A second mecha-

nism controlling or limiting the induction or transformation of potential antibody-forming cells to antibody-forming cells would account for this finding.

The plaque-forming cell response to the antigen was not detectable during the 1st week but rose to adult levels by the 5th or 6th week of extrauterine life. Hence, a very rapid growth of potential antibody-forming cells occurred during this time. The tolerant rats had a plaque-forming cell and antibody response during the 3rd week of life equivalent to that of rats of similar age which had received a single antigen injection; but quantitatively, this response was much lower than in adult animals receiving similar antigen injections. Continued antigen injections in the growing animals produced a decline and stabilization of this relatively small population of antibody-forming cells. Passively administered antibody profoundly inhibited the plaque-forming cell and antibody response of growing rats. It seems likely, therefore, that antibody actively produced by this small population of antibody-forming cells in tolerant rats inhibited the response of the growing population of potential antibody-forming cells, in a manner similar to that produced by passive administration of antibody.

Previous findings also support the concept that the production of antibody in small quantities may limit the antibody response. Unresponsiveness to sheep erythrocytes induced in adult rats by a single passive immunization was sustained for several months by weekly injections of sheep erythrocytes (3). The inhibitory effect of the passive immunization lasted less than 2 weeks. Therefore, the suppression was presumably sustained by antibody produced actively by relatively few antibody-forming cells. Furthermore, adult rats were made unresponsive to a large dose of sheep erythrocytes by initial active immunization with small doses of the antigen; unresponsiveness was sustained by repeated antigen injections (2). Again, unresponsiveness presumably resulted from inhibition of potential antibody-forming cells by antibody produced by relatively few antibody-forming cells stimulated by the initial small dose of antigen.

Suppression of the antibody response, whether in tolerant rats or rats made unresponsive by passive or active immunization, presumably occurs when a critical balance exists between the numbers of antibody-forming cells and the amount of antibody produced. Sufficient antibody has to be produced to block potential antibody-forming cells from responding to subsequent antigenic stimulation. On the other hand, the numbers of antibody-forming cells can not be so numerous as to produce appreciable circulating antibody to subsequent antigenic stimulation. Clearly, the concept of tolerance developed in these studies demands an active immune response with the production of antibody in small quantities for the maintenance of tolerance.

SUMMARY

An active immune response to sheep erythrocytes was demonstrated in rats made "tolerant" to sheep erythrocytes by twice-weekly antigen injections be-

ginning on the day of birth. Groups of tolerant rats were sacrificed 4 days after they had received 5 to 42 antigen injections; spleens were sampled for plaque-forming (antibody-releasing) cells and sera were titrated for antibody to sheep erythrocytes using a sensitive "plate hemolysin" technique.

During the 3rd week of life and after the 5th antigen injection, the tolerant rats had an immune response equivalent to that of rats of similar age which had received a single antigen injection, but spleens contained only about one-tenth as many plaque-forming cells as adults animals receiving similar antigen injections. Continued antigen injections produced a marked decline and stabilization of this relatively small population of antibody-forming cells; however, the number of plaque-forming cells in the tolerant rats remained considerably elevated above the numbers of plaque-forming cells present in the spleens of non-immunized animals. The sera from all but 1 tolerant rat had demonstrable antibody to sheep erythrocytes in low titer. A progressive recovery of the plaque-forming cell response and rise in antibody titers occurred in adult tolerant rats when the interval between the last 2 antigen injections was increased from 3 days to 14 or 28 days.

The decline and stabilization of numbers of plaque-forming cells occurring with continued injections after the 3rd week of life paralleled a similar decline and stabilization in rats receiving similar antigen injections as adults. Also, the recovery of the plaque-forming cell and antibody response of tolerant animals paralleled the recovery observed when the interval between injections was increased in rats receiving similar antigen injections as adults. These findings suggested that the same mechanism controlled numbers of antibody-forming cells in tolerant and normally responsive adult animals. Repeated closely spaced antigen injections presumably interfered with either cell division or maturation of antibody-forming cells. As the interval between injections was increased, additional antibody-forming cells matured or were formed through cell division. Relatively constant antigenic stimulation provided a mechanism for controlling or limiting the response of antibody-forming cells.

The mechanism controlling or limiting the response of antibody-forming cells would not account for the stabilization of numbers of antibody-forming cells at high levels for normal animals and at low levels for the tolerant animals. Passive immunization of growing rats with homologous anti–sheep erythrocyte serum markedly inhibited the plaque-forming cell response of growing rats. It was proposed that antibody produced by the small population of antibody-forming cells in the tolerant rats provided a feedback or homeostatic mechanism which inhibited transformation of potential antibody-forming cells to antibody-forming cells. Thus, tolerance to sheep erythrocytes was induced and maintained by two mechanisms. One mechanism, dependent on relatively constant antigenic stimulation, limited or controlled the numbers of antibody-forming cells. The other, dependent on the production of small quantities of antibody

by a few antibody-forming cells, limited or controlled the transformation of potential antibody-forming cells to antibody-forming cells.

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