REGULATION OF THE IMMUNE RESPONSE

I. Differential Effect of Passively Administered Antibody on the Thymus-Derived and Bone Marrow-Derived Lymphocytes*

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The production of humoral antibody to sheep erythrocytes (SRBC)[1] after the primary immunization of mice has been shown by a number of workers to be suppressed by the presence of passively administered anti-SRBC serum during the immunization (1–3 and for review see 4).

The development of antibody-producing or plaque-forming cells (PFC) in response to SRBC is a complex event both in vivo and in vitro involving a cooperation between several cell populations. One population, the thymus-derived lymphocytes (T-cells), is distinguished by the thymus-specific surface antigen, theta (5, 6). A small portion of these cells have receptors specific for determinants on the SRBC (SRBC-specific T-cells) and appear to be identical to a fraction of the SRBC rosette-forming cells (3, 7–9). The number of these cells increases markedly in the spleens of mice after immunization with SRBC (3, 7, 10, 11). T-cells have been implicated in the phenomenon of immunological memory (3, 7, 12, 13). There is a great weight of evidence that T-cells are involved in cellular immune responses, and very recently Cerottini et al. have shown that the cytotoxic activity of immune lymphocytes is abolished by pretreatment with anti-θ-serum and complement (14).

A second population of cells involved in the response to SRBC, generally referred to as the bone marrow-derived lymphocytes (B-cells) (for review see [15]), contains precursor cells with receptors specific for SRBC determinants (SRBC-specific B-cells) (8). After interaction with antigen and SRBC-specific T-cells these precursors divide, rapidly developing into PFC producing antibody specific for SRBC determinants (anti-SRBC PFC) (10, 16–18). It is the development of these PFC from precursors that is suppressed by passively administered anti-SRBC serum.

There is some evidence that the antigen-stimulated increase in T-cells is not nearly

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[1] *Abbreviations used in this paper:* B-cells, bone marrow-derived lymphocytes; HRBC, horse red blood cells; PFC, plaque-forming cells; SRBC, sheep red blood cells; T-cells, thymus-derived lymphocytes; TNP, trinitrophenyl(ated).

as sensitive as the development of antibody-producing cells to suppression by antiserum. Priming for phenomena which involve cellular immunity, such as delayed hypersensitivity, can be relatively insensitive to antiserum (19), as can priming for immunological memory (3, 20). In addition, Greaves et al. have shown that the antigen-stimulated increase in the SRBC rosette-forming cells of mouse spleens is less susceptible to suppression by anti-SRBC serum than is the development of anti-SRBC PFC (3).

Kettman and Dutton have described a method which allows the quantitation of SRBC-specific T-cells on the basis of their function, i.e., their ability to participate in the development of B-cell precursors into PFC (11, 21). Spleen cell suspensions cultured with the trinitrophenyl (TNP) hapten coupled to the carrier SRBC (TNP-SRBC) not only develop PFC specific for SRBC determinants but also PFC specific for TNP. As in the case of SRBC-specific B-cells, TNP-specific B-cells require the cooperation of T-cells in order to develop into anti-TNP–PFC. The participating T-cells in this case, however, show a specificity which is largely if not entirely for the SRBC carrier and are indistinguishable from the SRBC-specific T-cells which cooperate in the development of anti-SRBC–PFC. SRBC-specific T-cells are limiting in the spleens of normal mice, as shown by the greatly enhanced anti-TNP response of spleens from mice which have been primed with SRBC carrier. The degree of enhancement becomes an effective assay for their function.

Using this technique we have been able to show that anti-SRBC serum administered passively to mice in an amount sufficient to severely suppress the appearance of anti-SRBC–PFC in the spleen has little effect on the stimulation of SRBC-specific T-cells.

Materials and Methods

Mice.—8–12-wk old hybrid mice (BDF_1) from C57BL/6 female × DBA/2 male were used in all experiments. Both male and female mice were used, but within a given experiment mice of the same sex and age were used.

Antigens.—Sheep and horse erythrocytes (SRBC and HRBC) were obtained from the Colorado Serum Company, Denver, Colo. Trinitrophenylated erythrocytes were prepared by the method of Rittenberg and Pratt (22) as modified by Kettman and Dutton (21). Immunizations were performed by tail vein injection.

Antisera.—Mouse anti-SRBC serum was prepared by bleeding mice 10 days after the last of three weekly injections of 2×10^8 SRBC. A pool of antisera was made from 20 mice and used in all experiments reported here. The hemolytic titer of this pool against SRBC was 1/2048; the hemagglutination titer was 1/256.

Purified mouse anti-TNP antibody was the generous gift of Dr. Vincent Reed. It was isolated from hyperimmune mouse anti-TNP–hemocyanin serum by use of the immunoadsorbant TNP-Sephadex (23) and did not cross-react with SRBC.

Assay.—Cells producing antibody specific for SRBC determinants were enumerated by the Jerne hemolytic plaque assay (24) as modified by Mishell and Dutton (25). TNP-specific antibody-producing cells were assayed by the method of Rittenberg and Pratt (22) as modified by Kettman and Dutton (21) using TNP coupled to HRBC.

Cultures.—Spleen cell suspensions were cultured by the method of Mishell and Dutton (25), as modified by Kettman and Dutton (21), for the antigen TNP-SRBC.

RESULTS

Antisera, raised by repeated immunization with SRBC, were pooled from 20 mice and tested for the ability to suppress the primary humoral IgM response of mice to SRBC. The results of several experiments, shown in Table I, demonstrate that small amounts of the anti-SRBC serum administered passively to mice were very effective in preventing the appearance of IgM-producing anti-SRBC–PFC in the spleen. Virtually complete suppression was obtained with 1 μl and 82% suppression with only 0.2 μl. This pool was used in all subsequent experiments reported here.

In order to see if passively administered anti-SRBC serum is also capable of preventing the antigen-induced increase in SRBC-specific T-cells, spleen cell suspensions from mice which had been primed with SRBC in the presence or absence of anti-SRBC serum were cultured with the antigen, TNP-SRBC. The results of several experiments are shown in Table II. As reported earlier by Kettman and Dutton (11), it can be seen that priming of mice with the carrier, SRBC, leads to a large increase in the number of SRBC-specific T-cells in the spleen within 3–4 days, as measured by the 4 to nearly 20-fold enhancement of the anti-TNP response in vitro. The presence of anti-SRBC serum during the priming did not diminish this enhancement, i.e., under the conditions of these experiments anti-SRBC serum is unable to prevent the antigen-stimulated increase in SRBC-specific T-cells.

There is, however, a body of evidence that the antigen dose required for the production of humoral antibody is much higher than that necessary for the stimulation of T-cells (3, 26). In fact Falkoff and Kettman have shown in this system that the optimal SRBC priming dose for an enhanced anti-TNP response in vitro is 100-fold less than the optimal dose for the production of anti-SRBC–PFC.[2] The inability of anti-SRBC serum to suppress the stimulation of SRBC-specific T-cells would be explained if the action of the antiserum was to lower the effective antigen dose in the animal to a level that is far below the optimal for PFC development but still well in the optimal range for T-cell stimulation.

To examine this possibility it was necessary to test the effect of anti-SRBC serum in a range of antigen that is already limiting for T-cells. Dose-response studies for T-cell stimulation were performed over a wide range of priming antigen doses in the presence and absence of passively transferred anti-SRBC serum. The results of a representative (one of four) experiment are shown in Fig. 1 *a* and *b*. As shown by Falkoff and Kettman,[2] the optimal SRBC priming

[2] Falkoff, R., and J. R. Kettman. Differential stimulation of precursor cells and carrier specific thymus-derived cell activity in the in vivo response to heterologous erythrocytes in mice. Manuscript in preparation.

dose for the stimulation of SRBC-specific T-cells, as measured in the enhanced in vitro anti-TNP response (Fig. 1 *b*), is much lower than that for PFC production (Fig. 1 *a*). In fact very low priming doses fail to produce any detectable anti-SRBC–PFC, but still effect considerable T-cell priming. The presence of

TABLE I

The Suppressive Effect of Passively Administered Anti-SRBC Serum on the Development of Anti-SRBC–PFC in Mouse Spleens In Vivo

Exp. No.	Anti-SRBC serum	Anti-SRBC–PFC/10^6 spleen cells on day 4 after immunization			Suppression
		Antigen only	Antigen plus antiserum	No antigen or antiserum	
	(μl)				(%)
M18	10.0	98 ± 26 (3)	0.3 ± 0.1 (3)	n.d.	>99
J21	10.0	474 ± 175 (5)	0.6 ± 0.6 (5)	0.5 ± 0.2 (4)	>99
J14	2.0	100 (2)	5 (2)	7 (2)	>100
J17	2.0	517 (2)	4 (2)	<0.4 (2)	>99
M18	2.0	98 ± 26 (3)	1.3 ± 4.7 (3)	n.d.	>98
J21	1.0	474 ± 175 (5)	16 ± 12 (5)	0.3 ± 0.2	97
M18	0.2	98 ± 26 (3)	18 ± 11 (3)	n.d.	82

Mice were injected with antigen (2×10^7 SRBC) or antigen plus the indicated dose of anti-SRBC serum. After 4 days the animals were sacrificed, spleen cells suspensions prepared, and the number of anti-SRBC–PFC/10^6 cells determined. In most experiments a background determination was also made on the spleens of mice which received neither antigen nor antiserum. The per cent suppression was calculated as the suppressed response minus the background divided by the normal response minus the background × 100. Limits are 95% confidence levels. The numbers in the parentheses are the numbers of mice/group. n.d. = not determined.

TABLE II

The Effect of Passively Administered Anti-SRBC Serum on the Priming of SRBC-Specific T-Cells

Exp. No.	Priming before culture	Anti-SRBC Serum	Anti-TNP-PFC/10^6 recovered spleen cells on day 4		
			Unprimed	Primed with antigen only	Primed with antigen and antiserum
	(*days*)	(μl)			
M18	4	10	n.d.	952	1285
M18	4	2	n.d.	952	1250
M3	3	2	54	538	430
J10	3	2	19	320	287
M24	4	2	180	733	2500
J14	4	2	180	840	2800
J17	4	2	325	2150	2950

Mice were injected with antigen (2×10^7 SRBC) or antigen plus anti-SRBC serum. After 3 or 4 days spleen cell suspensions were prepared from these primed and from normal, unprimed mice, and were cultured with the antigen, TNP-SRBC. After 4 days the No. of anti-TNP–PFC/10^6 recovered spleen cells was determined.

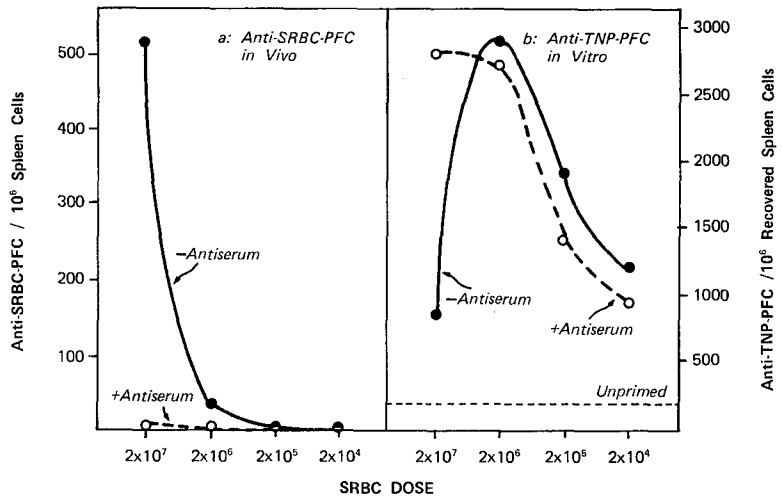

FIG. 1. Relation of PFC development, T-cell priming, and suppression by antiserum to antigen dose. Groups of two mice were injected with various doses of SRBC with (O) or without (●) 2 μl of anti-SRBC serum. (*a*) After 4 days the animals were sacrificed, pooled spleen cell suspensions prepared, and the number of anti-SRBC–PFC/10^6 cells determined. (*b*) The suspensions were cultured with the antigen, TNP-SRBC. After 4 days the number of anti-TNP–PFC/10^6 recovered cells was determined. The anti-TNP response of cultures from the spleens of unprimed mice is shown for comparison (-----).

TABLE III

The Suppressive Effect of Anti-TNP Antibody on the Development of Anti-SRBC–PFC and Anti-TNP–PFC In Vivo

Immunized with	PFC/10^6 spleen cells on day 4 after immunization	
	Anti-SRBC	Anti-TNP
(*a*) TNP-SRBC	243 ± 72 (5)	36 ± 25 (5)
(*b*) TNP-SRBC + anti-TNP	39 ± 36 (5)	2.6 ± 2.6 (5)
(*c*) TNP-SRBC + SRBC + anti-TNP	278 ± 103 (5)	1.6 ± 0.6 (5)

Groups of mice were injected as follows: (*a*) 2×10^7 TNP-SRBC; (*b*) 2×10^7 TNP-SRBC and 4 μg anti-TNP antibody; (*c*) 2×10^7 TNP-SRBC, 2×10^7 SRBC, and 4 μg anti-TNP antibody. After 4 days the animals were sacrificed, spleen cell suspensions prepared, and the number of anti-SRBC and anti-TNP–PFC/10^6 cells determined. Limits are 95% confidence levels. The numbers in the parentheses are the numbers of mice/group.

anti-SRBC serum during the priming severely suppresses the development of anti-SRBC–PFC (Fig. 1 *a*) but has little effect on the enhancement of the in vitro anti-TNP response (Fig. 1 *b*), indicating that even at a dose of antigen which is limiting, T-cell stimulation is not inhibited significantly by anti-SRBC serum.

It is interesting to note in Table II and Fig. 1 that at the relatively high

antigen dose of 2×10^7 SRBC the presence of anti-SRBC serum during the priming apparently improved the stimulation of SRBC-specific T-cells. The implications of this observation are considered in the discussion.

A number of mechanisms have been proposed to explain the suppression of the production of humoral antibody by passively transferred antiserum (for review see reference 4). Table III gives data from an experiment which bears on the evaluation of these mechanisms. Mice were immunized with TNP-SRBC. The ability of anti-TNP antibody to suppress the appearance of PFC specific for either TNP or SRBC determinants was tested. As the data show, anti-TNP antibody effectively suppresses the production of both anti-TNP–PFC and anti-SRBC–PFC. However, if free SRBC is injected together with the TNP–SRBC, the anti-TNP–PFC are still suppressed but not the anti-SRBC–PFC. In other words, antibody to a single determinant on an erythrocyte is a necessary and sufficient condition for the suppression of the response to other determinants of the erythrocyte.

DISCUSSION

Kettman and Dutton have recently reported a method which allows the in vitro detection of SRBC-specific T-cells (11, 21). The stimulation of mouse spleen cell suspensions with the antigen, TNP–SRBC, leads to the development of anti-TNP–PFC. A successful response is dependent on the cooperation of SRBC-specific T-cells with TNP-specific B-cell precursors. Spleen suspensions from mice which have been previously immunized with SRBC yield a greatly enhanced number of anti-TNP–PFC in vitro due to the increased number of SRBC-specific T-cells. The system provides a method for the assay of these T-cells on the basis of their function.

Using this technique spleens from mice which had been immunized 3–4 days previously with SRBC in the presence or absence of passively transfered anti-SRBC serum were assayed for SRBC-specific T-cells. The data show that a dose of antiserum which severely suppresses the appearance of anti-SRBC–PFC in the spleen (Table I) does not suppress the antigen-dependent stimulation of SRBC-specific T-cells (Table II).

These findings are consistent with data from other laboratories. In general, priming for immunological memory is more difficult to suppress by the passive transfer of antiserum than is the primary humoral response (3, 20). Priming for phenomena involving cellular immunity, such as delayed hypersensitivity, can be relatively insensitive to suppression by antibody (19). In addition, Greaves et al. have recently shown that in mice the antigen-dependent increase in the SRBC-specific rosette-forming cells of the spleen is less susceptible to suppression by anti-SRBC serum that is the development of anti-SRBC–PFC (3).

However, it has also been demonstrated that the antigen dose necessary for T-cell stimulation is much lower than that necessary for a humoral response.

Delayed hypersensitivity and immunological memory can be initiated with doses of antigen which fail to elicit a humoral antibody response (3, 26). Greaves et al. have obtained increases in the number of SRBC-specific rosette-forming cells of the spleen with antigen doses far below that necessary for the development of anti-SRBC–PFC. Finally and most relevant to the results reported here, Falkoff and Kettman have obtained significant stimulation of the SRBC-specific T-cells, as measured by the enhanced in vitro anti-TNP response, with very low SRBC priming doses.[2]

It was, therefore, possible that the relative insensitivity of T-cell priming to antiserum could be explained if the passively administered antiserum lowered the effective concentration of the antigen in the animal to a level below that necessary for PFC development but still within the optimal range for T-cell priming.

To test this possibility the effect of antiserum was determined in an antigen dose range that was already limiting for T-cell stimulation. Any further lowering of the antigen level in the animal caused by the antiserum would now be expected to have a marked effect on the stimulation of T-cells. That anti-SRBC serum does not prevent the stimulation of SRBC-specific T-cells even in this limiting antigen dose range is clearly shown by the results in Fig. 1, leaving the escape of T-cells from suppression as yet unexplained.

It is interesting to note in Table II and Fig. 1 that with a priming dose of 2×10^7 SRBC the stimulation of SRBC-specific T-cells is apparently improved by the presence of anti-SRBC serum during the priming. One explanation for this finding is the possible existence of competition between B-cells in their cooperation with T-cells in the in vitro assay. At this high antigen dose the production of anti-SRBC–PFC in the spleen indicates an expansion of the SRBC-specific B-cell population. This expanded population may compete effectively in vitro with the TNP-specific B-cells for the available SRBC-specific T-cells, thus lowering the anti-TNP response. The presence of antiserum during the priming prevents the expansion of the SRBC-specific B-cells and reduces the possible competition. Further data concerning B-cell competition for T-cells is reported by Falkoff and Kettman.[2]

A number of mechanisms have been proposed to explain the suppressive activity of antiserum on antibody-producing cells (for review see reference 4). They can be classified into three general types, any one of which may be modified to include the lack of suppression of T-cell stimulation. There is contradictory evidence for and against each of these mechanisms and it may be that depending on such factors as the physical properties or concentration of antigen, the class or dose of transferred antiserum, the species of animal, etc., any or all of the mechanisms may be operating in a particular experiment.

The first group of mechanisms depends on the regulation of antibody-producing cells by their own products, analogous to the feedback regulation seen

in many enzyme systems. In this scheme antibody or an antigen-antibody complex is capable of delivering a suppressive signal to antigen-specific B-cells which can be distinguished from the signal from antigen alone. This type of mechanism has been proposed by Rowley and Fitch (27) on the basis of the transfer of suppression into irradiated rats with cells which have been pretreated with anti-SRBC serum, and by Feldmann and Diener (28) on the basis of the inability of mice spleen cell suspensions to respond to the SRBC or bacterial flagellin in vitro or in irradiated recipients after an in vitro pretreatment with antigen and specific antiserum. The insusceptibility of T-cells could be explained by their inability to recognize the suppressive signal.

A second mechanism involves the covering of the antigenic determinants with the suppressing antiserum, thus isolating them from the B-cell precursors. This type of mechanism has been proposed by a number of workers in several experimental systems based on the ability of antiserum directed against certain determinants on a molecule or cell to specifically suppress the response to those determinants without affecting the response to other determinants on the same molecule or cell (29–31). The ability of T-cells to escape suppression may be explained in such a mechanism by their competing more efficiently than B-cells with the suppressing antiserum for the antigenic determinants or perhaps by their being directed against different determinants than the B-cells.

The third mechanism explains the suppressive effect of antiserum by its causing the rapid elimination of the antigen into phagocytic cells by opsonization. There is a considerable body of evidence in a number of experimental systems documenting the necessity of 7S (2) cytophilic antibody (32), with an intact Fc portion (33) of the molecule, for effective suppression. In addition, the participation of the macrophage in suppression has been demonstrated (34). The lack of inhibition of T-cell stimulation could be due to either its interaction with the antigen before elimination or by a continued access to the antigen after phagocytosis.

Evaluating these mechanisms on the basis of the results of the experiment reported in Table III, the third mechanism emerges as the only one consistent with the data. The ability of antibody directed against TNP to suppress the development of anti-SRBC–PFC in response to the antigen TNP-SRBC argues against a suppressive mechanism which requires the specific covering of antigenic determinants, or a specific feedback mechanism involving antibody alone. In addition, the specific prevention of the suppression of anti-SRBC–PFC by free SRBC argues against a suppressive feedback signal by an antigen-antibody complex. Although not conclusive, we feel that the data presented favor the elimination of antigen by opsonization as the primary means of suppression of the development of PFC in the experiments reported here.

It is as yet unclear how the antigen-stimulated increase in SRBC-specific T-cells escapes suppression by antibody. It may be that the mitogenic stimulation

of T-cells by antigen occurs even in the presence of antibody, as discussed in the above mechanisms. However, it is possible that the rapid increase in SRBC-specific T-cells in the spleen which follows immunization is not entirely due to proliferation, but is in part also due to the antigen directed "homing" of already existing T-cells to lymphoid tissues. Such a homing mechanism would depend on the channeling of antigen into the spleen and other lymphoid organs, a process which might be expected to be improved rather than hindered by antibody. The evaluation of this possibility awaits further evidence on the role of proliferation in the generation of T-cells in primed mice.

SUMMARY

The effect of passively transfered antiserum against sheep erythrocytes (SRBC) on the antigen stimulated increase of SRBC-specific plaque-forming cells (anti-SRBC–PFC) and SRBC-specific thymus-derived lymphocytes (SRBC-specific T-cells) in the mouse spleen was examined. A dose of antiserum which severely suppressed the development of anti-SRBC–PFC did not prevent the increase in SRBC-specific T-cells, as measured by their ability to cooperate in the in vitro response to trinitrophenylated (TNP) SRBC. It was shown that the insensitivity of these T-cells to antiserum could not be explained by their low antigen requirement as compared to that of PFC.

In the in vivo response of mice to TNP–SRBC, antibody specific for TNP suppressed the appearance of both anti-TNP– and anti-SRBC–PFC. The presence of free SRBC specifically prevented the suppression of the anti-SRBC–PFC. These observations are consistent with opsonization by phagocytic cells as the primary means of the observed suppression of PFC development by antibody.

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