SURFACE ANTIGENS OF IMMUNOCOMPETENT CELLS

III. In Vitro Studies of the Role of B and T Cells in Immunological Memory

By J. J. MOND,* T. TAKAHASHI,‡ and G. J. THORBECKE§

(From the New York University Medical Center, New York 10016)

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The immune response of dissociated murine spleen cells in vitro is a useful tool in the characterization of cellular events leading to antibody formation (1). A number of investigators have shown that antisera to immunoglobulins (Ig) interfere with the immune response of mouse spleen cells to sheep erythrocytes (SE)[1] in vitro (2–6). Since the antibody had to be left with the cells throughout the culture period, it was difficult to determine which cell type was affected by the anti-Ig. It would seem likely that the bone marrow-derived or B cells, having many Ig molecules on their surface (7–10), would be most readily affected by the anti-Ig. In fact, Takahashi et al. (11) have found that a large percentage of lymphoid cells in the murine spleen and lymph nodes, presumably B cells, can be killed by exposure to anti-Ig in the presence of complement. There is, in addition, some suggestive evidence regarding the presence of Ig molecules on the surface of thymus-derived or T cells (8, 12, 13). It is therefore of interest to study the functional activity of anti-Ig-incubated T and B cells separately.

Cell transfer studies in these and other laboratories have presented evidence for immunological memory in both T and B cells (14–16). It has since then been found that incubation with anti-Ig and complement is more effective in reducing the subsequent immune responsiveness of sensitized spleen cells in vitro than the one seen upon transfer to syngeneic recipients. Therefore, the effectiveness of anti-Ig-treated cell populations in restoring anti-θ-treated cells could be studied more readily by in vitro methods.

The present studies demonstrate that, indeed, anti-Ig-treated immune T cells complement anti-θ-treated immune B cells in vitro. They establish the specific-

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[1] *Abbreviations used in this paper:* BA, *Brucella abortus*; B cells, bone marrow-derived cells; C′, complement; GPC′, guinea pig complement; HBSS, Hanks' balanced salt solution; HGG, human gamma globulin; M199, medium 199; NMS, normal mouse serum; NRS, normal rabbit serum; PFC, plaque-forming cells; RC′, rabbit complement; SE, sheep erythrocytes; T cells, thymus-derived cells.

ity of "memory T cells" and provide information on the development of these cells with time after immunization.

Materials and Methods

Mice.—Male BALB/c mice 3–6 months old were obtained from Cumberland View Farms, Clinton, Tenn. Immunization was by intravenous injection of 10^7 or 10^6 SE, or 2.5×10^7 killed *Brucella abortus*[2] organisms (BA), or 1.0 mg of human gamma globulin (HGG).[3] Spleens were removed for tissue culture 3–14 days after immunization. All suspensions for tissue culture were prepared from pools of two to six mice.

Antisera Preparations.—Alloantiserum detecting θ-C3H was prepared in (AKR/*H*-2^b × A/θ-AKR)F_1 against A-strain leukemia ASL1 according to methods previously described (17). Another anti-θ-C3H serum was prepared by immunization of AKR mice (Jackson Laboratory, Bar Harbor, Maine) with C3H thymus cells. Both antisera were cytotoxic for >90% of thymus cells at dilutions of 1:5000. Anti-immunoglobulin (Ig) serum with specificity for both H and L chains was prepared by immunizing rabbits with mouse Ig according to methods described by Potter (18). Reactivity of the anti-Ig with L chains was determined by double diffusion in agar, employing mouse L chains kindly donated by Dr. K. R. McIntire (National Cancer Institute, Bethesda, Md.). Normal rabbit serum (NRS) and anti-Ig sera were absorbed extensively with BALB/c thymocytes in order to remove natural cytotoxic antibody against mouse cells (11).

Treatment of Spleen Cell Suspensions.—Spleen cells were obtained by gentle teasing into Hanks' balanced salt solution (HBSS). All cells were washed twice and then treated with antisera as follows. 3.0 ml of immune spleen cells at a concentration of 10^7 cells/ml in medium 199 (M199, Microbiological Associates, Inc., Bethesda, Md.) were added to a test tube containing equal volumes of appropriate dilutions of complement (C′) and of control serum or antiserum. Dilutions (prepared in M199) for the reagents were: rabbit C′, 1/15 (RC′); guinea pig C′ 1/4 (GPC′); mouse sera, 1/12 (NMS, anti-θ) unless indicated otherwise; rabbit sera, 1/15 (NRS or anti-Ig). Mouse antisera were used in combination with RC′ and rabbit antisera with GPC′. The sera used for C′ activity were previously screened for lack of natural heteroantibody to mouse thymus cells.

Cells were incubated for 45 min at 37°C with intermittent gentle mixing, washed twice, resuspended in RPMI 1640 + 10% fetal calf serum (Associated Biomedic Systems, Buffalo, N.Y.), and the contents of each tube were divided over two or three culture dishes. In reconstitution experiments, normal or untreated immune spleen cells were added to the dishes together with the preincubated cells. Cells were cultured according to the method of Mishell and Dutton (1) in the presence of 10^7 SE.

Assay for Plaque-Forming Cells (PFC).—After 3–4 days of culture, cells were collected and washed once in HBSS. The number of direct PFC was determined by the method of Jerne et al. (19) and expressed as PFC per dish.

RESULTS

Secondary Responses of Different Numbers of Immune Spleen Cells In Vitro

In experiments aimed at showing a specific effect of removing defined populations of cells from cell mixtures, it is of importance to establish the influence of

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[2] The *Brucella abortus* test antigen was kindly donated by Dr. C. B. Watson from the U.S. Department of Agriculture.

[3] Human gamma globulin was generously provided by the American Red Cross.

total cell numbers on the degree of responsiveness. Therefore, experiments were performed with graded numbers of immune spleen cells per dish. It was found that 10^7 immune spleen cells performed equally as well as 2×10^7, and frequently even slightly better. Lowering the cell numbers to 5×10^6 rarely lowered the PFC response as can be seen in Table I. Further diminution of cell numbers invariably reduced the response significantly and at cell numbers less than 10^6 per dish very few, if any, PFC were obtained. Other results indicated that addition of 5×10^6 or 10^7 normal spleen cells to 1.5×10^6 or more immune spleen cells always reduced the response of such immune spleen cells. Addition of normal spleen cells to 5×10^5 immune spleen cells either did not affect or very slightly increased the response of such cells alone. With the RPMI-1640 medium and culture conditions employed in the present experiments normal spleen cells at levels of 5×10^6 or 10^7 per dish gave either very low (<500 PFC) or no primary response to SE in vitro.

TABLE I

The Secondary Response to SE In Vitro by Various Concentrations of Mouse Spleen Cells

No. of cells per dish ($\times 10^6$)	No. of PFC per dish*				
	Experiment 1 (day 4)‡	Experiment 2 (day 4)	Experiment 3 (day 6)	Experiment 4 (day 7)	Experiment 5 (day 7)
10	21,400	45,000	6780	26,000	22,000
5	31,500	44,000	6240	18,000	23,200
2.5	5800	11,600	2840	7400	13,000
1.5	700	1400		6900	4000
0.5	0	170		0	0

* No. of PFC were determined on day 3 (experiments 2 and 3) or 4 after initiation of culture.

‡ Day after intravenous injection of 10^7 SE at which spleen cells were taken for culture.

Reconstitution of Secondary Response after Depletion of θ-Bearing Cells.— Incubation with RC′ and anti-θ at a final dilution of 1/36 reduced the responsiveness of immune spleen cells to 1–6% (Table II, experiments 6, 8, and 9). Higher dilutions of the anti-θ (to 1/75) still reduced the response to approximately 20% of control values. Addition of twice the number of anti-θ-treated cells per dish gave no higher response. Addition of $5–10 \times 10^6$ normal spleen cells gave slight reconstitution in experiment 6, and none in experiments 7–9. Similar numbers of normal thymus cells or 2.5×10^6 normal lymph node cells also failed to reconstitute (not in tables). This result confirms previous observations with transferred immune spleen cells and suggests the presence of immunological memory in T cells (14, 15).

As few as 0.5×10^6 untreated immune spleen cells gave a significant degree of reconstitution of responsiveness to the anti-θ-treated cells of the same spleen, while addition of 1.5×10^6 cells gave complete reconstitution in three of four ex-

periments (Table II). It should be noted that 0.5–1.5 × 10^6 immune spleen cells alone or combined with normal spleen cells gave much lower responses. The values of those responses were always subtracted from the reconstituted values given in Tables II and III, and were usually much lower than the numbers of PFC obtained from the cell combinations (<5%).

It appeared of interest to determine whether the ability to reconstitute the T cell memory to SE would be present at early time intervals after immunization. Since it had been suggested that T cells require relatively low doses of antigen for activation (20), a low primary dose of 10^6 SE was chosen and the

TABLE II

Comparison of the Abilities of Normal and Immune Spleen Cells to Reconstitute the Secondary Response of Anti-θ-Treated Spleen Cells*

Preincubated‡ cells	Spleen* cells added	No. of PFC per dish				
		Experiment 6	Experiment 7	Experiment 8	Experiment 9	Experiment 10
NMS + C′	None	18,000	5270	18,500	33,000	28,000
Anti-θ + C′	None	900	1550	160	970	6600
Anti-θ + C′	Normal, 10 × 10^6	2600	1260	120	920	
Anti-θ + C′	Normal, 5 × 10^6	3200	630		1260	
Anti-θ + C′	Immune, 1.5 × 10^6	21,400§	5400	6600	‖	24,300
Anti-θ + C′	Immune, 0.5 × 10^6			140	6910	19,500

* Spleen cells were taken on day 4 (experiment 9), 7 (experiments 6 and 8), or 10 (experiment 7) after intravenous injection of 10^7 SE.

‡ Immune spleen cells were incubated with anti-θ + rabbit C′ for 45 min at 37°C.

§ Numbers of PFC obtained from the combination of cells after subtraction of numbers of PFC given by reconstituting cells alone. Subtracted values were as follows: experiment 6 (4000), experiment 7 (0), experiment 8 (1600 and 0), experiment 9 (1540), experiment 10 (6900 and 90).

‖ Background by 1.5 × 10^6 cells alone as high as reconstituted value of 14,000.

spleen cells taken 3–4 days afterwards. It was found, as seen in Table III, that 0.5 × 10^6 of these cells gave a significant degree of reconstitution. Comparison between Tables II and III shows that addition of 1.5 × 10^6 cells of the 3–4-day immune spleen cells to the anti-θ-treated cells resulted in complete reconstitution similar to that obtained with immune spleen cells taken on day 7 after SE injection. In contrast, spleen cells taken on day 14 after SE injection did not reconstitute nearly as efficiently as did the cells taken early after immunization although they were still much more effective than normal spleen cells. Further studies are in progress to follow the appearance and persistence of these memory T cells upon immunization with various antigen doses.

The specificity of this phenomenon was also investigated. The results in Table III suggest that 2.5 × 10^6 *B. abortus* or HGG immune spleen cells, taken

4 days after intravenous immunization, are not effective in reconstituting the the response of the anti-θ-treated cells to SE. Other results from this laboratory however, suggest that higher numbers of cells immunized to other antigens may under certain conditions be significantly more effective than normal spleen cells in reconstituting the response of anti-θ-treated SE-immune spleen cells to SE.[4]

Reconstitution of Secondary Response after Depletion of Ig-Bearing Cells.— Killing of Ig-bearing cells with anti-Ig and GPC′ also resulted in decreased

TABLE III

Relative Abilities of Spleen Cells Immune to SE or Other Antigens to Reconstitute the Secondary Response of Anti-θ-Treated Spleen Cells

Preincubated* immune spleen cells	Cells‡ added	No. of PFC per dish			
		Experiment 5	Experiment 8	Experiment 10	Experiment 11
NMS + C′	None	23,200	18,500	28,000	5400
Anti-θ + C′	None	3200	160	6600	100
Anti-θ + C′	Days 3–4 immune 0.5×10^6	9600§	4800	20,000	1700
Anti-θ + C′	Days 3–4 immune 1.5×10^6	21,600	4800	24,300	3100
Anti-θ + C′	Day 14 immune 0.5×10^6		40		420
Anti-θ + C′	Day 14 immune 1.5×10^6	7060	260		2200
Anti-θ + C′	Day 4 BA immune 2.5×10^6	2000			
Anti-θ + C′	Day 4 HGG immune 2.5×10^6			5200	

* Immune spleen cells, taken 7–10 days after intravenous injection of 10^7 SE, were preincubated with NMS or anti-θ and rabbit C′ for 45 min at 37°C.

‡ "Days 3–4 immune" spleen cells were taken 3 (in experiments 8 and 11) or 4 days (experiments 5 and 10) after intravenous injection of 10^6 SE. "Day 14 immune" spleen cells were taken 14 days after intravenous injection of 10^7 SE. "BA immune" and "HGG immune" spleen cells were taken 4 days after intravenous injection of 2.5×10^7 killed *B. abortus* organisms, or 1.0 mg human γ-globulin, respectively.

§ Numbers of PFC obtained by combinations of cells after subtraction of numbers of PFC formed by reconstituting cells alone. Subtracted values were 20% (experiment 5) and <5% (experiments 8, 10, and 11) of the reconstituted values.

responsiveness, leaving 7–40% (average 29% for eight experiments) of the response seen in control dishes (Tables IV and V). In order to show that the lower response was not due to decreased numbers of viable cells in culture (approximately 0.8×10^7 cells/dish), additional dishes were included which received twice as many anti-Ig-treated cells. The response in such dishes was even less than with the lower cell number.

Attempts to reconstitute this depleted response with $5–10 \times 10^6$ normal

[4] Mond, J. J., T. Takahashi, and G. J. Thorbecke. 1972. T cell activation by heterologous antigens as a replacement of specific immune T cells in the transfer of the secondary response to sheep erythrocytes. *J. Exp. Med.* **136**:715.

spleen cells failed. This was also observed when anti-Ig-treated immune spleen cells were transferred to syngeneic mice (15), and was interpreted as showing immunological memory in Ig-bearing cells.

Complementation of Anti-θ-Treated with Anti-Ig-Treated Immune Spleen Cells.— Table V and Fig. 1 show clearly that virtually complete reconstitution of the

TABLE IV

Inability of Normal Spleen Cells to Reconstitute the Secondary Response of Anti-Ig-Treated Immune Spleen Cells In Vitro*

Preincubated cells*	No. of normal spleen cells added	No. of PFC per dish		
		Experiment 6	Experiment 12	Experiment 13
NRS + C′	None	16,500	2500	4250
Anti-Ig + C′	None	1320	500	1350
Anti-Ig + C′	10×10^6	3800		1240
Anti-Ig + C′	5×10^6	3400	420	1510

* Immune spleen cells, taken 7 days after intravenous injection of 10^7 SE were incubated with NRS or anti-Ig (1/45), and GPC′ (1/12) for 45 min at 37°C.

TABLE V

Reconstitution of Normal Responsiveness In Vitro by Recombination of Anti-θ-Treated and Anti-Ig-Treated SE Immune Spleen Cells

Immune spleen cells	% of PFC response of control dishes				
	Experiment 6	Experiment 12	Experiment 14	Experiment 15	Experiment 16
NS-treated cells*	100 (16,500)	100 (2000)	100 (2000)	100 (18,400)	100 (11,500)
Anti-Ig-treated‡	7	33	12	36	40
Anti-θ-treated§	5	9	12	60	2
Anti-Ig-treated + Anti-θ-treated	75	75	90	100	75

* Control dishes received either NRS + GPC′ or NMS + RC′ incubated cells. Results in parentheses represent average numbers of PFC per dish for both controls.

‡ Immune spleen cells were incubated with anti-Ig + GPC′ for 45 min at 37°C.

§ Immune spleen was incubated with RC′ and anti-θ 1/36 in experiments 6, 16; 1/75 in experiments 12, 14; 1/300 in experiment 15 for 45 min at 37°C.

secondary response was obtained when anti-θ-treated immune spleen cells were reconstituted with anti-Ig-treated cells from the same spleen. This observation demonstrates that the two antisera affect different cell populations. Reconstitution was not always to 100% of control values. This may have been because of the fairly high viable cell numbers in dishes with cell combinations, as compared with 1.0 or 1.5×10^7 in dishes with control cells.

DISCUSSION

The presence of immunological memory in T cells has been shown by a variety of experimental approaches. (*a*) Carrier-sensitized T cells specifically mediate the ability of B cells to respond to hapten-protein conjugates in vivo (21, 22) and in vitro (23, 24). (*b*) The reduction in the secondary immune response resulting from the removal of T cells from sensitized lymphoid cells can be restored by much lower numbers of specifically sensitized than of normal cells or of cells sensitized to noncross-reacting antigens (16, 25). This is also clearly established in the present findings showing a relative inability of normal thymus, lymph node, or spleen cells to reconstitute the depleted secondary responses to SE

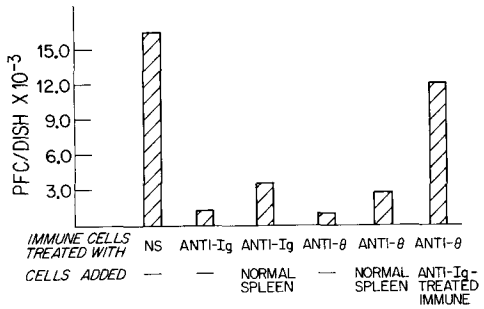

FIG. 1. Results of a representative experiment expressed as numbers of PFC per dish obtained with immune mouse spleen cells after 3 days of culture in the presence of SE. Normal rabbit serum and guinea pig C′-treated cells (NS) were used to determine the control level of the responses. Note the low degree of reconstitution of the responses obtained by adding 5×10^6 normal spleen cell to anti-Ig or anti-θ-treated cells. In contrast to this is the marked degree of reconstitution seen after addition of anti-Ig-treated immune spleen to anti-θ-treated immune spleen.

in vitro of anti-θ-treated immune spleen cells. (*c*) "Educated" T cells are more effective than normal T cells in inducing an immune response in bone marrow cells in vitro (26) and in vivo (27). (*d*) Immune T lymphoid cells have an enhanced ability to mediate such typical T cell functions as transfer of delayed hypersensitivity (28) and specific in vitro cytotoxicity (29, 30).

Although these findings appear to establish a specific memory function of T cells, it should be realized that nonspecific enhancement of B cell function has also been accomplished by a variety of means in vivo (31–34) and in vitro (33, 35–39). It is, therefore, important to study the reconstituting effect of various doses of cells sensitized to other antigens on the response of anti-θ-treated SE-immune spleen cells. In the present studies the specificity of the reconstituting T cells has been established by the relatively large effect of extremely low numbers of SE-sensitized cells as compared with the ineffectiveness of similar numbers of cells sensitized to *B. abortus* or HGG but cultured in the absence of their sensitizing antigen.

The present studies, however, do not establish whether T cell and B cell interaction is most effective when both cell types are directed against the same antigen, or whether activated T cells of any specificity can, in the presence of their sensitizing antigen, enhance B cell responses. In the latter case a soluble mediator induced by activated T cells may be involved, and would be obtained by reexposing sensitized T cells to their sensitizing antigen (35) or else by exposing T cells to nonspecific mitogens or to *H-2* antigens (32, 38, 39). It should be noted, that the observation on the phenomenon of nonspecific B cell activation by allogeneic cells, suggesting that a graft-vs.-host but not a host-vs.-graft reaction is needed (32, 34), speak against a primary role for soluble factors.

A more thorough investigation of the cross-reactivity between antigens at the level of the T cell is also needed in view of results of other investigators suggesting differences in specificities between B and T cells in this regard (20, 27). Further studies are underway in this laboratory to compare the relative ability of T cells sensitized to various antigens in the presence and absence of their sensitizing antigens to restore the responses of anti-θ-treated SE-immune spleen cells.

The present data show large differences between SE-immune spleen cells taken at varying intervals after immunization in their ability to reconstitute the anti-θ-treated cells. The concentration of sensitized T cells appears to be higher in spleen cells taken on days 3–7 than on days 10–14 after SE injection. It may be that the memory T cells are relatively sparse in the spleen after the longer interval because they have migrated to other lymphoid tissues. A semiquantitative evaluation of T cell memory at different times after immunization as well as after varying immunization procedures can be obtained by this type of experimental approach. The present results suggest, for instance, that 5×10^5 immune spleen cells may comprise more SE-immune T cells than do $5–10 \times 10^6$ normal cells.

There is ample evidence for the presence of immunological memory in B cells (15, 16, 21, 22, 40) which are the cells that in the mouse (11) and in the chicken (41) can be killed by exposure to anti-Ig and C′. The present experiments demonstrate that normal spleen cells cannot reconstitute the secondary immune response of anti-Ig-treated spleen cells to SE in vitro. Similar results were previously obtained in spleen cell transfer experiments (15). Preliminary observations in this laboratory show that small numbers of spleen cells from appropriately immunized mice are capable of reconstituting the depleted response of anti-Ig-treated cells. Evidence has also been obtained showing that combinations of 5×10^6 spleen cells from mice taken 15 days after SE injection with 5×10^5 spleen cells from 4-day immune mice give much higher responses than could be expected from a simple additive effect of the responses by these two populations when cultured separately. In view of the relative lack of reconstituting T cells in the 15-day spleen, it would seem likely that there is a high propor-

tion of immune B cells in these spleens which cooperate with T cells from the early immune spleen.

Since anti-θ-treated immune spleen was virtually completely reconstituted in its ability to give a secondary response to SE in vitro by addition of anti-Ig-treated immune spleen cells, the anti-Ig did not appear to affect the θ-bearing primed T cells. Two recent publications have appeared in agreement with this finding showing a lack of inhibition of T cell function by anti-Ig in the primary response to SE in vitro (42) and in the T cell-mediated specific target cell killing (43).

Nevertheless, there is some evidence in the literature that T cells have an Ig receptor (44–46). Most of these results have been obtained with the use of specific antisera to kappa chains. Lesley et al. (47) found an inhibitory effect of such an antiserum on T rather than on B cells, employing a similar experimental approach to the one reported here. In addition, Greaves et al. (46) showed that anti-L chain inhibited the mixed lymphocyte reaction between human lymphoid cells in vitro and that the effectiveness of the antiserum was neutralized by absorption with L chains. In contrast, in this laboratory mixed lymphocyte reactions between rabbit cells were not found to be inhibited by anti-kappa chain allotype antiserum (48). In experiments by Mason and Warner (49) the ability of cells to transfer delayed hypersensitivity in the mouse could be inhibited by previous exposure of the cells to some anti-L chain antisera in vitro in the absence of C′. A similar inhibition was obtained by anti-Ig on the effectiveness of cells to induce graft-vs.-host reactions (50, 51). Evidence that immunocompetent thymus cells bind antigen was obtained from "suicide" experiments using highly radioactive protein antigen (13). This phenomenon was inhibited by simultaneous addition of anti-L chain.

In the present experiments the anti-Ig was able to kill B cells while T cells were neither killed nor blocked by exposure to this antiserum. It seems possible that T cells have a different kind of Ig receptor than do B cells and that the anti-Ig used in the present experiments reacts only with B cells, even though it did contain antibody to L chain as well as to μ and γ chain. Since it appears that only some, but not all, anti-L chain sera are active in inhibiting T cell function (49), the specificity of the antisera should be further examined before definite conclusions about the nature of the T cell receptor can be drawn.

SUMMARY

The effect of preincubation with anti-θ or anti-mouse immunoglobulin (Ig) and complement (C′) on immune responsiveness of spleen cells from BALB/c mice immunized with sheep erythrocytes (SE) was investigated. Both treatments greatly depressed the remaining ability to produce a secondary response to SE in vitro.

Normal BALB/c spleen cells were far less effective in reconstituting the

responses of such depleted cell populations than were much smaller numbers of untreated immune spleen cells. Thymus-derived cell (T cell) memory appeared early after immunization and showed specificity for the immunizing antigens.

Recombination of anti-Ig-treated with anti-θ-treated immune spleen cells resulted in virtually complete reconstitution of responsiveness. The presence of immunological memory in T cells and the nature of their surface receptors are discussed.

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