THYMUS-DERIVED CELL (T CELL) ACTIVATION BY HETEROLOGOUS ANTIGENS AS A REPLACEMENT OF SPECIFIC IMMUNE T CELLS IN THE TRANSFER OF THE SECONDARY RESPONSE TO SHEEP ERYTHROCYTES*

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A few reports have appeared which suggest that bone marrow-derived cell (B cell)[1] precursors can be induced to form antibody after stimulation by antigen in the absence of thymus-derived cells (T cells). With some antigens, such as pneumococcal polysaccharides and *Brucella abortus*, this appears to represent a complete or relative T cell independence of the antibody response (1, 2). In T cell-dependent immune responses, such as those to sheep erythrocytes and hapten conjugates, the T cell function or carrier effect can be replaced by nonspecific stimuli. The nonspecific stimuli used were injection of allogeneic cells in vivo (3, 4) or addition of such cells in vitro (5), injection of polyribonucleotides (6, 7), or addition in vitro of cells responding to other antigens (8, 9).

The present studies were performed to study whether a replacement of specific T cell function can be obtained in vivo by T cells immune to and activated by another unrelated antigen. The *B. abortus* antigen was chosen for these studies because it is known to stimulate even normal thymus cells very effectively (10) and because it does not cross-react with sheep erythrocytes.

Materials and Methods

Donor BALB/c mice (Cumberland View Farms, Clinton, Tenn.), 3–6 months old, were immunized by intravenous injection of 10^6 or 10^8 sheep erythrocytes (SE) and 10 μg of *Escherichia coli* endotoxin (Difco Laboratories, Inc., Detroit, Mich.), 10^8 killed *B. abortus* organisms (BA) (kindly donated by Dr. C. E. Watson from the U. S. Dept. of Agriculture), or 1.0 mg of keyhole limpet hemocyanin (KLH). Recipient mice received 650 R whole body

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[1] *Abbreviations used in this paper*: B cells, bone marrow-derived cells; BA, *Brucella abortus*; C′, rabbit complement; KLH, keyhole limpet hemocyanin; ME, mercaptoethanol; NMS, normal mouse serum; PFC, plaque-forming cells; SE, sheep erythrocytes; T cells, thymus-derived cells.

γ-irradiation (Gammator M; Radiation Machinery Corp., Parsippany, N.J.) 1 day before cell transfer.

Alloantiserum detecting θC3H was prepared by immunization of AKR mice (Jackson Laboratory, Bar Harbor, Maine) with C3H thymus cells (11). The antiserum was cytotoxic for >90% of thymus cells at a dilution of 1:5000 in the presence of rabbit complement (C′).

Spleen cells were obtained by gentle teasing into Hanks' balanced salt solution. Cells were washed and treated with antiserum as follows. Immune spleen cells, at a concentration of 10^7 cells/ml in medium 199 (Microbiological Associates, Inc., Bethesda, Md.), were added to a test tube containing equal volumes of C′ (1/15) and of alloantiserum to θ or normal mouse serum (1/12). The C′ was previously screened for lack of natural heteroantibody to mouse

TABLE I

Reconstitution of the Transferred Immune Response of Anti-θ-Treated SE Immune Spleen by Immune or Normal Spleen Cells*

Reconstituting cells added	Preincubated immune spleen*	Indirect PFC‡ per recipient spleen
None	NMS + C′	127,100
None	Anti-θ + C′	1000
Immune spleen 10^6 (long term)*	Anti-θ + C′	79,900
	None	5160
Immune spleen 10^6 (day 3)§	Anti-θ + C′	22,700
	None	0
Normal spleen 10^7	Anti-θ + C′	1420
	None	680

* Immune spleen taken from mice 30 days after the last of four biweekly intravenous injections of 20% SE (0.1 ml). Preincubation with anti-θ (1/45) and rabbit C′ (1/45) was for 50 min at 37°C and at a concentration of 3×10^6 cells/ml. Recipients received 5×10^6 cells intravenously.

‡ Assayed on day 6 after transfer. Expressed as average number of indirect PFC per spleen (four mice) after subtraction of the numbers obtained for direct PFC.

§ Taken on day 3 after intravenous injection of 0.1 ml of 0.2% SE.

thymus cells. Cells were incubated for 50 min at 37°C, washed twice, and injected intravenously into mice at a dose of 5×10^6 cells per recipient. "Reconstituting" cell suspensions and antigens were mixed with the preincubated cells immediately before injection.

Recipient mice were exsanguinated on day 6 after transfer of cells. Their spleens were assayed for both direct and indirect plaque-forming cells (PFC) to SE (2). Sera were titrated for agglutinin titers to SE by doubling dilutions. Sensitivity to mercaptoethanol (ME) was determined after incubation of 1:5 diluted serum with an equal volume of 0.2 M ME for 1 hr at room temperature.

RESULTS

As shown previously (2, 12) anti-θ treatment greatly diminished the immune response of the hyperimmune spleen cells to SE (Tables I and II). A significant degree of reconstitution of this immune responsiveness was obtained with as few as 10^6 untreated SE immune spleen cells (Table I). Of particular interest

was the high degree of reconstitution by cells taken 3 days after a single injection of a very low dose of SE, which when transferred alone did not give rise to any indirect PFC. In contrast, even 10 times as many normal spleen cells did not cause a significant degree of reconstitution as was also shown previously in transfer studies (2). These results agree with those obtained in experiments on the in vitro secondary response to SE (13).

TABLE II

Reconstitution of the Transferred Immune Response of Anti-θ-Treated SE Immune Spleen by Syngeneic Spleen Cells from Mice Immunized to other Antigens*

Immune spleen* preincubated with	Reconstituting cells added	Antigen	Indirect PFC per recipient spleen*			
			Exp. 1	Exp. 2	Exp. 3	Exp. 4
NMS + C′	None	SE + BA§	33,000	12,500	20,100	16,000
Anti-θ + C′	None	SE + BA§	1190	130	0	880
Anti-θ + C′	BA immune spleen day 7, 10^7 cells	SE + BA	6320	6450	10,060	5700
		SE	380	1790	1670	1700
Anti-θ + C′	BA immune spleen day 3, 10^7 cells	SE + BA	4100	4680	2050	
		SE	240	10	540	
Anti-θ + C′	KLH immune spleen day 7, 10^7 cells	SE + KLH				4400
		SE				140

* Immune spleen was taken from mice 14–30 days after the last of two to three biweekly intravenous injections of 20% SE (0.1 ml) and 10 μg *E. coli* endotoxin. Preincubation with anti-θ (1/45) and rabbit C′ (1/45) was for 50 min at 37°C and at a concentration of 3×10^6 cells/ml. Recipients received 5×10^6 cells intravenously.

‡ Assayed on day 6 after transfer. Expressed as average number of indirect PFC per spleen (four mice) after subtraction of the numbers obtained for direct PFC. Because the reconstituting cells gave very low direct and no indirect PFC when given alone, these values were also subtracted and are not represented separately in the table.

§ Addition of BA did not influence the response of NMS or anti-θ-treated cells to SE.

‡ In experiment 1 an additional group of recipients received anti-θ-treated BA immune spleen along with the anti-θ-treated SE immune spleen. The average PFC per spleen obtained was 410.

Although 10^7 normal spleen cells did not reconstitute the ability of anti-θ-treated spleen cells to give a secondary response to SE, whether or not BA was also injected, a similar number of spleen cells from BA immune mice did cause a variable degree of reconstitution when given together with both the antigens. In three consecutive experiments (not in the tables) the indirect PFC response was reconstituted to a level of 23, 40, and 100% of the response seen with control immune spleen cells.

In order to determine whether the BA antigen cross-reacted to a large degree

with SE at the T cell level or whether activation of BA immune T cells by the BA antigen was causing this enhancing effect, the experiments represented in Table II were carried out. It was established in experiment 1 that anti-θ treatment of the day 7 BA-sensitized spleen abolished its reconstituting effect. In addition, the enhancing effect obtained in all the experiments was much greater in the presence of BA antigen than in its absence. This suggested that the effect was due to the activation of T cells from the reconstituting population by BA.

Kruger and Gershon (10) have shown that BA antigen as well as KLH are excellent stimulators of thymus cell proliferation. Therefore, an additional experiment was performed using 10^7 KLH-sensitized spleen cells, taken 7 days after injection of 1 mg of KLH, and the results were similar to those obtained with BA. It should be noted that adding BA to the challenge injection of normal mouse serum (NMS) or anti-θ-treated SE-hyperimmune cells never influenced the height of the response obtained to SE (cf. reference 2). Injection of endotoxin (*E. coli*, 10 μg) together with SE at the time of challenge also failed to reconstitute the response of anti-θ-treated spleen cells.

The response seen in these experiments included a direct PFC response which varied from approximately 15 to 50% of the indirect PFC obtained. The degree of reconstitution was slightly more difficult to judge for this response, because reconstituting cells gave some direct PFC on their own, but in general followed exactly the same pattern as the indirect PFC. The serum agglutinin 7S and 19S titers against SE also agreed in principle with the results obtained for PFC per spleen. The over-all serum titers were always several-fold increased when BA-sensitized spleen and BA were given together with anti-θ-treated memory cells, but after mercaptoethanol treatment these differences were less pronounced. Only in those instances where the number of 7S PFC were substantially reconstituted was there also a detectable increase in the ME-resistant serum antibody. This was probably due to the fact that the animals were killed on day 6 after transfer, a time at which splenic indirect PFC were just reaching peak values, while the 7S serum antibody titers were only beginning to rise.

DISCUSSION

These results suggest that specific SE-sensitized T cell function can be at least partially replaced in the immune response to SE by T cells sensitized to and activated by another unrelated antigen. Such activated cells are far more effective than normal T cells or than T cells sensitized to another antigen but transferred without reinjection of that antigen. Because the highest response left after anti-θ treatment amounted to 5% of the control response and $<1\%$ was left in some experiments, it seems unlikely that the replacement effect of T cells was obtained by stimulation of surviving SE-sensitive T cells. Rather it appears that a stimulatory effect of the BA- or KLH-activated T cells on the SE-sensitized B cells was obtained. The sensitivity of the reconstituting cells to anti-θ treatment showed that this effect was mediated by T cells. This

suggests that a humoral factor is released by activated T cells which helps in stimulating B cell proliferation and differentiation, both with respect to the 19S and the 7S immune responses.

A similar conclusion was reached by investigators studying the replacement of T cell function by polyanions (7, 14, 15). The release of a soluble mediator into culture supernatant from activated thymus-derived (16–18) or thymus cells (19, 20) was shown by its effect on other cultured cells. Thymus extracts or soluble factors released by thymus have also been shown to cause partial reconstitution of thymectomized animals in vivo (21–23). However, some of the data from the literature also suggest that thymus cells together with a humoral factor are far more effective than is humoral factor alone (24). A few other observations also indicate that a humoral factor cannot completely replace the requirement for specific T and B cell interaction: (*a*) The present studies show that specific SE-sensitized T cells, even in $\frac{1}{10}$ the number as the unrelated activated T cells, are much more effective in reconstituting the response of anti-θ-treated SE-immune spleen. (*b*) Results of others (25, 26) show that antigens, which have both the determinants reacting with immune T and immune B cells on the same molecule, are more effective in inducing a secondary response by B cells than are mixtures of antigens, although these mixtures should react with the two cell types and thus be equally good at activating the T cells. (*c*) Allogeneic cells can replace the carrier effect in the secondary response of the host to hapten conjugates only when a graft-*versus*-host rather than a host-*versus*-graft reaction is incurred (3).

It should be pointed out that a completely different problem from the one discussed here is that of cross-reactivity between antigens which might be stronger for some antigens at the T cell than at the B cell level (27–30). Because the response to sheep erythrocytes is not enhanced by prior immunization to BA of the same animal, this does not appear to apply here. Nevertheless a small reconstituting effect was obtained in some experiments with the BA-sensitized cells in the absence of a specific BA challenge. It seems likely that this effect was sometimes seen simply as a result of intrinsic T cell activation in the transferred BA-immune spleen cells, possibly related to carry-over of persisting antigen.

SUMMARY

Spleen cells from LAF_1 mice hyperimmune to sheep erythrocytes (SE) lost their ability to transfer a secondary response to irradiated recipients after incubation with anti-θ and rabbit complement in vitro. Small numbers of specific immune cells even when taken 3 days after a primary SE injection reconstituted the direct and indirect plaque-forming cell responses.

Larger numbers of cells sensitized to *B. abortus* (or keyhole limpet hemocyanin), and given together with the corresponding antigen, also partially reconstituted the ability to respond to SE. This property was mediated by

θ-bearing cells and was interpreted as due to a nonspecific humoral factor liberated by specifically activated T cells and acting on B cell proliferation or maturation.

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