INDUCTION OF A HEMOLYSIN RESPONSE IN VITRO

II. Influence of the Thymus-Derived Cells During the Development of the Antibody-Producing Cells*

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It has been shown that thymus-derived cells play an important role during the development of a humoral immune response; thus, newborn thymectomized mice (1) or bone marrow chimeras (2, 3) responded normally to injected immunogens only after transfer of intact thymus cells. There is evidence, derived from transfer experiments with so-called "educated thymus cells" (4) and from experiments with "tolerant" thymus cells (5, 6), that the thymus-derived cells are antigen-sensitive, that they have to recognize the immunogen specifically to be able to assist the humoral immune response; nevertheless, most of the antibody-secreting cells do not develop from the thymus cell population as demonstrated by chromosomal analysis (7, 8) and histocompatability antigen typing (1). It might be suggested that the thymus-derived cells are the carrier-recognizing cells. Their number is crucial for the magnitude of the immune response (9), but little is known about their function, when they act during the onset and development of the immune response, and how they assist the triggering of the antibody precursor cells or the expansion of the antibody-secreting cell clones. It is not even known if their presence and function is essential or only facilitating the immune response.

The development of a hemolysin response, the formation of PFC,[1] has been studied also in spleen cell cultures (10). Treatment of the spleen cell suspension with anti-theta serum impairs the immune response (11); the response could be restored by addition of educated thymus-derived cells (12). Furthermore the hemolysin response in vitro was impaired when spleen cells from newborn thymectomized mice (13) or from bone marrow chimeras (14–17) were taken into culture; again, the response could be restored by addition of irradiated spleen cells or by thymus cells.

[1] *Abbreviations used in this paper:* B cells, spleen cells from bone marrow chimeras; BSS, Hanks' balanced salt solution; HRBC, horse red blood cells; PFC, direct local hemolysin-producing cells; SRBC, sheep red blood cells; T cells, spleen cells from irradiated and thymus cell-grafted animals.

The experiments reported here were undertaken to learn more about the role of the thymus-derived cell. Cell suspensions were made from the spleens of thymectomized, irradiated, and bone marrow-grafted mice; this was the source of the bursa equivalent (B) cells containing most of the antibody precursor cells (17). The thymus derived (T) cells were obtained from spleens of irradiated and thymus cell-injected mice. The T cells assisted the development of the hemolysin response only when the thymus cells had been injected into the irradiated hosts together with immunogenic erythrocytes. Since it is not yet clear if selection, multiplication, or differentiation takes place during the time in the host to the antigen-sensitive cells, this process was circumscribed as "education." These educated T cells were able to assist the development of PFC in the presence of the erythrocytes used for education (17). In the present study the influence of the number of educated T cells on the number of the direct PFC during the development of the hemolysin response was investigated.

Materials and Methods

Animals.—BDF₁ mice (C57BL/6 female × DBA/2 male), 6–12 wk old, were used in all experiments.

B Cell Suspension.—6-wk old mice had been thymectomized; 1 wk later they were irradiated (850 R) and grafted with 3×10^7 isogeneous bone marrow cells (always mice of the same sex were used as donors and acceptors of the cells). 15–25 days later these mice were killed by cervical dislocation and the spleen removed and made into suspensions (10, 17). The mediastinum of each mouse was controlled for macroscopical absence of thymus tissue.

Educated T Cells.—Mice had been irradiated (700 R) and injected intravenously with 5×10^7 isogenous thymus cells and 10^7 sheep (SRBC) or horse (HRBC) red blood cells, respectively, at the same day. 8–10 days later the mice were killed, the spleens removed and made into suspensions (17). In several experiments controls for PFC in these spleen cell suspensions were performed; no PFC were detected.

Cultures.—Spleen cells were obtained by gentle teasing in Hanks' balanced salt solution (BSS); the cells were washed twice (centrifuged at 100 g), and resuspended in complete medium (Eagle's minimal essential medium [MEM] from Microbiological Associated Inc., Bethesda, Md., enriched with glutamine, Na-pyruvate, nonessential amino acid mixture, antibiotics, and 5% fetal calf serum from Reheis Chemical Co., Chicago, Ill., lot no. 268). The cell suspensions were cultivated in plastic Petri dishes (Falcon plastics, Los Angeles, Calif., No. 3001) according to the methods of Mishell and Dutton (10) in the presence of immunogenic erythrocytes. These cultures were incubated in an atmosphere of 7% O_2, 10% CO_2, and 83% N_2; they were kept on a Bellco rocking platform. Every day 100 μl of feeding cocktail (10) was added.

Cell Harvest and Assay for Hemolysin-Producing Cells (PFC).—The cells were harvested after 3–5 days in culture. The number of PFC per culture or per 10^6 recovered, nucleated cells was determined by a modification of the technique described by Jerne et al. (18) and Mishell and Dutton (10). The experiments were repeated many times, within one experiment the numbers of PFC were counted from two or three cultures which were harvested separately.

RESULTS

Cooperation Between Bone Marrow-Derived Spleen Cells and Educated T Cells.—In these experiments B cells (suspended spleen cells of thymectomized,

irradiated, and bone marrow-grafted mice) or SRBC-educated T cells (suspended spleen cells of mice which had been irradiated and injected with thymus cells and sheep erythrocytes) were kept in culture and stimulated with SRBC under the conditions described by Mishell and Dutton (10); none or only few PFC could be detected after 3–5 days in culture. When the B cells and T cells were mixed and cultured together in the presence of SRBC, then the development of many PFC could be observed.

The number of PFC was dependent on the concentration of the added T cells; an increased number of educated T cells to a constant number of B cells led to an increased number of PFC detectable 3 or 4 days later (Table I) which was directly proportional to the number of T cells added to the cultures.

TABLE I

Cooperation Between Different Numbers of Educated T Cells and Bone Marrow-Derived Spleen Cells In Vitro

Spleen cell suspensions		PFC/culture (in parenthesis PFC/10^6 recovered cells)
B cells	T cells	
(Exp. 335)		
4×10^6	—	15 (8)
4×10^6	+ 0.5 × 10^6	360 (200)
4×10^6	+ 1.0 × 10^6	500 (283)
4×10^6	+ 2.0 × 10^6	710 (309)
4×10^6	+ 4.0 × 10^6	1980 (637)
—	4.0 × 10^6	80 (80)

B cells: Mice were thymectomized, irradiated (850 R), and grafted with 3×10^7 bone marrow cells 3 wk before the experiment.

T cells: Mice were irradiated (700 R) and injected with 5×10^7 thymus cells and 10^7 SRBC 9 days before the experiment. The cells were cultured together with SRBC; PFC were assayed 4 days later.

Increase in the Number of PFC During the Culture Period.—B cells and educated T cells were mixed and distributed evenly in several culture dishes. Immunogenic erythrocytes were added and 3 or 4 days later three cultures of each combination were harvested and assayed for the number of PFC (Table II), in the presence of higher concentrations of educated T cells a greater increase in the number of PFC between the 3rd and 4th day of culture became visible.

Increase in the Number of B Cells in the Cultures.—The number of PFC detectable in the cultures was also dependent on the concentration of the B cells (Table III); increasing the concentration of the B cells by a factor of three led to an almost threefold increase in the number of PFC per culture. This increase was difficult to detect if the PFC were calculated per 10^6 harvested nucleated cells. This may have been due to the fact that educated T cells do not survive

as well as B cells in culture, and as a result, probably constituted the minority population of cooperating cells in the system.

Addition of Educated T Cells to Normal Mouse Spleen Cells.—When spleen cells of 5–7-wk old BDF₁ mice (of our inbred colony) were maintained 4 days in culture together with immunogenic erythrocytes, the number of developing PFC was often rather low compared with the original results reported by Mi-

TABLE II

Rise of PFC in the Cultures of B Cells and Educated T Cells

Spleen cell suspensions		Immunogen	PFC/culture assayed with			
			SRBC		HRBC	
B cells	T cells		day 3	day 4	day 3	day 4
(Exp. 332)						
2.9×10^6	—	SRBC	27	10	—	—
2.9×10^6 +	1.2×10^6 (I)	SRBC	100	190	—	—
2.9×10^6 +	3.6×10^6 (I)	SRBC	235	1060	—	—
—	3.6×10^6 (I)	SRBC	0	0	—	—
(Exp. 333)						
5.0×10^6	—	HRBC	—	—	16	0
5.0×10^6 +	1.5×10^6 (II)	HRBC	—	—	100	420
5.0×10^6 +	4.5×10^6 (II)	HRBC	—	—	200	3040
—	4.5×10^6 (II)	HRBC	—	—	0	0
(Exp. 337)						
5.5×10^6	—	SRBC	65	60	—	—
5.5×10^6 +	0.2×10^6 (I)	SRBC	160	160	—	—
5.5×10^6 +	0.6×10^6 (I)	SRBC	290	520	—	—
5.5×10^6 +	2.0×10^6 (I)	SRBC	580	1490	—	—
1.8×10^6 +	0.2×10^6 (I)	SRBC	50	60	—	—
1.8×10^6 +	0.6×10^6 (I)	SRBC	120	250	—	—
—	2.0×10^6 (I)	SRBC	15	5	—	—

B cells: Mice were thymectomized, irradiated (850 R), and grafted with 3×10^7 bone marrow cells 17 (exp. 332), 19 (exp. 337), or 30 (exp. 333) days before the experiment.

T cells: Mice were irradiated (700 R), injected with 5×10^7 thymus cells and 10^7 SRBC (group I) or 10^7 HRBC (group II), respectively, 9 days before preparation of the spleen suspensions. PFC were assayed 3 and 4 days later.

shell and Dutton (10), or with spleen cell cultures from older mice. Addition of educated T cells to these normal spleen cells led to an increase in the number of PFC in these cultures (Table IV).

Influence of Educated T Cells on the Response to Heterologous Erythrocytes.— In earlier experiments (17) it was observed that educated T cells, in the presence of the immunogen to which they had been preexposed, were also able to assist in the development of PFC against non–cross-reacting erythrocytes. This

assistance again is dependent on the number of added T cells; increasing the number of SRBC-educated T cells will also help the anti-HRBC response, and vice versa. However, although an increase in the number of PFC specific for the educating erythrocytes in the presence of educated T cells was observed

TABLE III

Cooperation Between B Cells and Educated T Cells During the Hemolysin Response In Vitro

Spleen cell suspensions		PFC/culture	PFC/10^6 cells
B cells	T cells		
(Exp. 347)			
4.4×10^6	—	0	0
1.3×10^6 +	0.16×10^6	80	150
4.4×10^6 +	0.16×10^6	180	163
1.3×10^6 +	0.50×10^6	210	430
4.4×10^6 +	0.50×10^6	600	481
1.3×10^6 +	1.50×10^6	680	850
4.4×10^6 +	1.50×10^6	1600	1070
—	1.50×10^6	0	0

B cells: Mice were thymectomized, irradiated (850 R), and grafted with 3×10^7 bone marrow cells 17 days before the experiment.

T cells: Mice were irradiated (700 R) and injected with 5×10^7 thymus cells and 10^7 SRBC 10 days before the experiment. The cells were cultured together with SRBC. PFC were assayed 5 days later.

TABLE IV

Interaction Between Normal BDF_1 Spleen Cells, and Educated T Cells In Vitro

Spleen cell suspension		PFC/culture (in parenthesis per 10^6 recovered cells) assayed with			
Normal spleen cells	T cells	SRBC		HRBC	
		day 4	day 5	day 4	day 5
(Exp. 346)					
6.5×10^6	—	420 (140)	1680 (600)	300 (100)	1480 (527)
6.5×10^6 +	0.4×10^6 (I)	1500 (705)	5760 (2060)	350 (176)	1080 (384)
6.5×10^6 +	1.6×10^6 (I)	2300 (744)	9360 (3120)	510 (164)	1600 (534)
—	1.6×10^6 (I)	0	0	0	0
6.5×10^6 +	0.5×10^6 (II)	950 (339)	1780 (494)	1920 (686)	10000 (2940)
—	0.5×10^6 (II)	0	0	0	0

T cells: Mice were irradiated with 700 R, injected with 5×10^7 thymus cells and 10^7 SRBC (group I) or 10^7 HRBC (group II), respectively, 9 days before preparation of the spleen cell suspensions. The cells were cultured together with sheep and horse erythrocytes. PFC were assayed 4 and 5 days later.

until the 5th day of culture, a corresponding increase in the number PFC specific for the non–cross-reacting erythrocytes was not detected (Table V).

DISCUSSION

The experiments were made in tissue cultures as described by Mishell and Dutton (10). Although others had observed some stimulating effect by the

addition of suspended thymocytes (15, 16), such cells were found to inhibit the response in our cultures (14, 17). Thus, it was necessary first to inject the thymocytes into lethally irradiated animals and later the spleens were taken as the source of thymus-derived cells; certainly other cells are also present in this suspension, e.g. the radioresistant cells. These cells did cooperate well during the immune response if immunogenic erythrocytes had been injected together with the thymocytes into the irradiated animals. When placed in the suspension cultures, these cells had been already exposed to the immunogen and are

TABLE V

Rise of PFC in the Cultures of B Cells Educated T Cells

Spleen cell suspensions			PFC/culture assayed with					
			SRBC			HRBC		
B cells	T cells		day 3	day 4	day 5	day 3	day 4	day 5
(Exp. 343)								
5×10^6	—		95	90	110	65	60	190
5×10^6	+	0.5×10^6 (I)	250	1310	2570	105	270	270
5×10^6	+	2.0×10^6 (I)	450	3000	4840	270	820	840
—		2.0×10^6 (I)	30	35	270	5	10	0
5×10^6	+	0.5×10^6 (II)	55	110	60	135	700	620
5×10^6	+	2.0×10^6 (II)	165	370	790	215	1420	5200
—		2.0×10^6 (II)	0	0	0	0	0	0

B cells: Mice were thymectomized, irradiated (850 R), and grafted with 3×10^7 bone marrow cells 16 days before preparation of the spleen cell suspension.

T cells: Mice were irradiated (700 R), injected with 5×10^7 thymus cells and 10^7 SRBC (I) or 10^7 HRBC (II), respectively, 8 days before the experiment. The cells were cultured together with SRBC and HRBC.

Cultures were harvested after 3, 4, and 5 days and assayed for PFC.

therefore referred to as educated T cells. No antibody-producing cells (direct PFC) develop from these educated thymus-derived cells. Addition of such educated T cells to anti-theta serum–treated spleen cell suspensions was found to restore the hemolysin response (12).

The B cells (bursa equivalent cells) were taken from mice which had been thymectomized, lethally irradiated, and injected with syngeneic bone marrow cells (without heterologous erythrocytes). The spleens of these animals were taken into suspension; again many different cells, radioresistant cells as well as various cells derived from the injected bone marrow, were present in this suspension. The precursor cells of the PFC were in this population (17).

The two cell suspensions were taken into cultures together and immunogenic erythrocytes were added. In these cultures the number of PFC detected 3 and 4 days later was dependent on the concentration both of the B cells and the educated T cells. In many experiments doubling of the concentration of the T

cells led to doubling of the detectable PFC (Table I) (this linear relationship was not always so clearly visible in the results however, probably because of other limiting cells or factors involved in the complicated culture system). From these data it is not yet possible to calculate the number of T cells involved since it is not known how many precursor cells will have been stimulated, or how often the precursor cell will have divided before the PFC assay. The concentration of the educated T cells does not only influence the absolute number of PFC but also their increase during the culture period; with higher concentrations of T cells the time for doubling the number of PFC becomes shorter. This faster increase of the number of PFC could be explained by continuous recruitment of more precursor cells (19, 20), but the experiments using different concentrations of B cells and the limiting dilution experiments in microcultures (unpublished data) suggest that the concentration of the T cell influences the size of the PFC clones as well as the number of clones. It seems that a high concentration of educated T cells promotes or influences positively the clone expansion.

Certainly the educated T cells are already needed at the start of the cultures; addition of the T cells 24 hr after culturing the B cells delayed the increase of the number of PFC. Furthermore, experiments to separate the cooperating cells by millipore membranes or in soft agar medium have failed so far to demonstrate an influence of soluble mediators on the T cells (unpublished data).

It was shown earlier (17) that educated T cells, if stimulated by the educating erythrocytes, will also assist the development of PFC to non-cross-reacting erythrocytes. This cannot be explained by cross-recognition on the T cell since only in the presence of the educating erythrocytes will these help the development of other PFC. The help is best seen in the presence of high concentrations of T cells (Table V); furthermore, the increase in the number of non-cross-reacting PFC ceases earlier than the response to the educating erythrocytes. This suggests that there is competition for the T cells, and that the B cells specific to the same immunogen are helped preferentially.

In the cultures of normal spleen cells the number of PFC could be increased by addition of educated T cells, but not by B cells. This suggests that in the spleen cell suspensions of our young mice the T cells are limiting the response. Here addition of educated T cells almost exclusively stimulates the increase of the PFC to the educating erythrocytes (Table IV). It seems that limited numbers of T cells preferentially help the B cells directed against the educating erythrocytes.

The interpretations of these experiments are not complete since the time of recruitment and the time of proliferation are not yet known (21). Nevertheless it seems justified from these data to conclude that the T cells influence not only the recruitment, the triggering event, but also the time of proliferation (possibly by allowing the proliferation to continue a longer time or by shortening

the time of division). It seems that the developing PFC clones need to stay in the neighborhood of the stimulated T cell, and that by limiting concentrations of T cells, at the start as well as during the development of the PFC, those precursor cells will be aided preferentially which carry the same specificity as the educated T cells. This preference could be established by specific binding to the immunogen. In this way the T cells might be involved in the hemolysin response, observing only the direct PFC in the response to particulate immunogens, not only at the onset of the response, but also later in regulating the size of the clones and the degree of response by their continuous presence or activity.

SUMMARY

Spleen cells of bone marrow chimeras (B cells) and of irradiated mice injected with thymus cells and heterologous erythrocytes (educated T cells) were mixed and cultured together (17). The number of PFC developing in these cultures was dependent both on the concentration of the B cells and of the educated T cells. In excess of T cells the number of developing PFC is linearly dependent on the number of B cells. At high concentrations of T cells more PFC developed; the increase in the number of PFC was greatest between the 3rd and 4th day of culture. Increased numbers of educated T cells also assisted the development of PFC directed against the erythrocytes. It is concluded that the T cells not only play a role during the triggering of the precursor cells but also during the time of proliferation of the B cells; close contact between B and T cells seems to be needed to allow the positive activity of the T cells.

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