SPECIFIC HETEROLOGOUS ENHANCEMENT OF IMMUNE RESPONSES

IV. Specific Generation of a Thymus-Derived Enhancing Factor*

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Recent experimental evidence has provided strong support for the concept that at least two distinct populations of lymphoid cells cooperate in the production of humoral antibody. Synergism between thymus-derived (T cell)[1] and bone marow-derived (B cell) lymphocytes has been described in the induction of antibody synthesis against heterologous erythrocytes (1–3), serum proteins (4, 5), and hapten-protein conjugates (6–8). Although both participating cell types can respond to specific antigenic stimulation (9, 10), actual synthesis of exportable immunoglobulin is apparently limited to cells of B lymphocyte lineage (11, 12). While T cells therefore do not secrete detectable amounts of antibody, they appear in some cases to facilitate antibody production by B cells. However, the mechanism of interaction between these components is not well defined.

We recently demonstrated that augmented immune responses similar to those after reexposure to the same antigen need not be specific (13). Inbred mice were primed with tetanus toxoid (TT); later, their spleen cells were maintained in culture with sheep erythrocytes (SRBC). The addition of TT (the antigen used initially for priming) to these cultures resulted in significant enhancement of the anti-SRBC plaque-forming cell (PFC) response as compared to aliquots cultured without TT. The addition of TT to comparable cultures of spleen cells from normal mice, or the addition of another antigen to TT-primed cultures, failed to produce enhancement of anti-SRBC plaques. Moreover, augmentation of the number of plaques was obtained by adding TT to mixed cultures of normal spleen cells plus thymocytes from TT-primed mice (14). These observations were consistent with the concept, first suggested by Davies et al. (11), that a mediator released from specifically stimulated T cells acts on the antibody-forming B cells to increase their productive response. Supernatants derived from

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[1] *Abbreviations used in this paper:* B cell, bone marrow-derived lymphocyte; T cell, thymus-derived lymphocyte; PFC, plaque-forming cell; SRBC, sheep erythrocytes; TT, fluid tetanus toxoid; FBS, fetal bovine serum; EF, enhancing factor; $ThyT^+$, supernatants from primed thymocytes cultured with tetanus toxoid; $ThyT^-$, supernatants from primed thymocytes cultured without tetanus toxoid; $SplTS^+$, supernatants from primed spleen cells cultured with tetanus and sheep erythrocytes; $SplT^+$, supernatants from primed spleen cells cultured with tetanus toxoid; Thy, thymocytes; Spl, spleen cells.

cultures of tetanus toxoid stimulated, TT-primed spleen cells were tested for such an effector molecule. We found that the cell-free culture fluid, when added to SRBC-stimulated, normal spleen cell cultures, significantly enhanced the anti-SRBC PFC response (15). This activity was termed enhancing factor (EF).

The purpose of this paper was to confirm and extend the finding that a humoral factor, released upon specific interaction of antigen with a primed population of T cells, can increase the antibody response to another antigen present in the system. In cell cultures containing SRBC-stimulated normal spleen cells, and TT-primed cells from either thymus or spleen, enhancement of the anti-SRBC PFC response occurred when 1 ng of TT was added, although tenfold fewer thymocytes than spleen cells were needed to produce comparable results. Furthermore, supernatants of cultures of thymocytes from TT-primed mice and TT were found to contain a soluble enhancing factor. It was absent from similar cultures of normal thymocytes plus TT, and from primed thymocytes cultured without TT.

Materials and Methods

Immunization of Mice.—8-wk old DBA/2 female mice (Jackson Laboratory, Bar Harbor, Maine) were immunized intravenously with 10 Lf of fluid tetanus toxoid (Massachusetts Department of Public Health, Division of Biologic Laboratories, Boston, Mass.). Mice were shown to be primed by finding antitoxin in their serum, as previously described (13).

Preparation of Spleen Cell Cultures.—Cell cultures were prepared from the spleens of immunized and normal mice 30–60 days after injection by the method of Mishell and Dutton (16) as modified by Mosier (17). Each culture contained 1 ml of cells (2×10^7) suspended in medium supplemented with 10% fetal bovine serum (FBS) (Reheis Chemical Co., Kankakee, Ill.). Cultures were fed daily with a nutritional supplement (13) and were stimulated with approximately 3 million SRBC on the day of culture (day 0). 1 ng of tetanus toxoid was added to half of these cultures on day 2. The in vitro dose of TT and the time of addition were needed to elicit the optimal enhancement effect (13). Some cultures received only the priming antigen in the absence of SRBC stimulation.

Preparation of Mixed Lymphoid Cultures.—Suspensions of TT-primed thymocytes and normal thymocytes were prepared as described previously (14). Briefly, the two-thirds of the thymic lobes distal to the hilus were excised, care being taken to avoid fascial tags which might contain lymph node tissue. All adherent connective tissue was removed. A number (approximately 10%) of the DBA/2 mice used as thymus donors were injected intraperitoneally with 0.2 ml of Pelikan ink (Günther Wagner, Hanover, Germany; lot No. C11/1431A) diluted 1:1 with saline, in order to reveal any parathymic nodes as found by Leckband and Boyse (18). None were found in DBA/2 mice. In contrast, in C3H mice treated similarly, parathymic lymph nodes were easily detected in the connective tissue lateral to the thymic hilus. Thymus lobes were teased apart and treated similarly to the spleen cell suspensions. Thymocytes were suspended in complete medium (13) to a final concentration of 20 million cells/ml. Experimental cultures contained 10 million normal spleen cells and 10, 1, or 0.1 million primed or normal thymocytes. Parallel mixed cultures containing spleen cells from both immunized and normal mice were also prepared. Mixed lymphoid cell cultures were stimulated with TT and SRBC as described above and direct "19S" plaques enumerated on day 5 by a modification of the method of Jerne and Nordin (13).

Preparation of Supernatants.—On day 5, the spleen cell culture fluid was collected and centrifuged to remove cells (200 *g*, 10 min, 4°C). Supernatants harvested from three to five

cultures per experimental group were stored at −20°C until assayed for enhancing activity. In addition, supernatants were prepared from short-term cultures of thymocytes from TT-primed and normal animals by the methods described above. Thymocyte cultures containing 20 million cells were stimulated with 1 ng of TT on day 0. Half of the cultures were unstimulated. Supernatants were collected sequentially from replicate cultures at 24, 48, and 72 hr and were treated as described earlier.

Evaluation of Supernatant Activity.—Supernatants from cultures of spleen cells or thymocytes were assayed for the ability to augment the anti-SRBC PFC response by methods previously described (15). Briefly, spleen cell cultures from normal mice were prepared and were stimulated with 3 million SRBC on day 0. Filtered supernatants, diluted in fresh medium, were added to such cultures on day 2. Direct plaques were counted on day 5.

The degree of enhancement is expressed as the per cent difference between the numbers of plaques produced in cultures that received test supernatants, compared to the PFC response of cultures that received the appropriate dilution of a normal control supernatant, i.e., derived from normal spleen (or normal thymocyte) cultures not receiving antigen. Normal control supernatants were used as base line controls to correct for any enhancing activity provided by FBS, by constituents released from dying cells, or produced by viable cells in response to FBS foreign antigenic determinants. Supernatants assayed for enhancing factor included those derived from TT-primed spleen cell cultures that received (*a*) both tetanus and SRBC ($SplTS^+$), or (*b*) tetanus toxoid alone ($SplT^+$). Supernatants from cultures of TT-primed thymocytes maintained in the presence or absence of toxoid were also tested for enhancing factor. These supernatants were designated $ThyT^+$ and $ThyT^-$, respectively. Comparable supernatants from cultures of unprimed, normal thymocytes were tested in parallel.

Heat Treatments.—$ThyT^+$ supernatants were tested for the stability of enhancing factor to mild heat treatment. One-half of each supernatant was heated at 56°C for 30 min. Each aliquot was then diluted and the activity assessed as described above.

Dialysis.—$ThyT^+$ supernatants were divided into two aliquots. One aliquot was dialyzed against 1000 volumes of isotonic saline for 24 hr at 4°C; an equal volume was kept cold, but not dialyzed. Treated and untreated aliquots were added to assay cultures and tested for the ability to amplify the anti-SRBC PFC response.

Enzyme Treatments.—Supernatants with enhancing activity ($SplTS^+$, $SplT^+$, and $ThyT^+$) were tested for their sensitivity to enzymatic degradation with deoxyribonuclease I (DNAse, lot No. 5613; Worthington Biochemical Corp., Freehold, N. J.), ribonuclease A (RNAse, lot. No. 100C-8062; Sigma Chemical Co., St. Louis, Mo.), or protease (type VI, lot No. 71C-9020; Sigma Chemical Co.). Supernatants were divided into two aliquots. DNAse, RNAse, or protease was added to one aliquot at a final concentration of 10 μg/ml. Supernatant-enzyme mixtures were mixed constantly (1 hr, 37°C) to ensure adequate contact between enzyme and substrate. Treated and untreated aliquots were then assayed for activity by the usual method.

RESULTS

Relative Numbers of Primed Thymocytes or Spleen Cells Needed to Produce Enhancement in Mixed Cell Cultures.—In a previous paper we reported that at least some components of antigen-specific immunological memory reside in the thymus of mice immunized 1–2 months earlier (14). Specific stimulation in vitro of thymocytes from TT-primed mice, cultured with SRBC-stimulated normal spleen cells, produced a significant increase in the anti-SRBC hemolysin response as measured by plaque formation (14). In order to quantitate the number of thymocytes necessary to produce this amplifying effect in vitro, cell mixtures containing 10 million normal spleen cells and graded numbers of

syngeneic thymocytes from mice immunized with TT were stimulated with SRBC and the specific priming antigen. A parallel study was performed substituting TT-primed spleen cells for the TT-primed thymocytes. The results of a representative group of experiments are shown in Table I. The addition of 1 ng of TT on day 2 to SRBC-stimulated, normal spleen cell cultures containing 10 million or 1 million TT-primed thymocytes produced an average of 67% and 89% enhancement, respectively, of the anti-SRBC PFC response compared with similar cultures without the priming antigen (groups 1, 2 and 5, 6). The addition of TT to parallel cultures containing similar numbers of normal spleen cells and normal thymocytes resulted in a 20% and 18% depression, respectively, in the anti-SRBC response (groups 3, 4 and 7, 8). Although 1 million primed thymocytes could be triggered in vitro to produce nonspecific enhancement of the PFC response, when the number of primed thymocytes was reduced to 1×10^5 per culture, the addition of TT failed to elicit an augmented PFC response (groups 9 and 10). Similar additions of priming antigen to positive control (groups 13 and 14) and negative control cultures (groups 15 and 16), from representative experiments, produced augmentation or slight depression, respectively, of the PFC response, as previously reported (13). Cultured thymocytes alone, stimulated with TT and SRBC, failed to produce any plaques (13). In parallel experiments, in which 10 million TT-primed spleen cells were substituted for the 10 million primed thymocytes, the addition of 1 ng of toxoid resulted in a degree of augmentation of the anti-SRBC PFC response comparable to that seen in Table I (groups 1 and 2, Table II). However, in contrast to the enhancement seen in mixtures of 1 million primed thymocytes and 10 million normal spleen cells, the addition of priming antigen to cultures with similar numbers of primed spleen cells and normal spleen cells resulted in a 14% depression in the anti-SRBC response (groups 5 and 6, Table II). Enhancement was consistently observed in representative positive controls, (groups 9 and 10), while slight depression was seen in the negative controls (groups 11 and 12).

Release of Enhancing Factor from Thymocytes.—From the data in Table I it is apparent that the addition of the specific priming antigen to primed thymocytes maintained in the presence of normal spleen cells resulted in the nonspecific augmentation of the in vitro immune response to a non-cross-reacting (13) antigen. It was of interest to determine whether the mechanism of this enhancement phenomenon was the same as that previously reported for specifically stimulated spleen cells (15), i.e., a humoral factor released by the specific interaction of antigen with a primed population of T cells can increase the productive antibody response of B cell lymphocytes reacting to a heterologous antigen. Since thymocytes maintained in the absence of a supporting lymphoid cell population do not survive for extended periods of time in vitro[2] (19), we cultured TT-primed and normal T cells from the thymus for 24–72 hr.

[2] Rubin, A. S. Unpublished observations.

TABLE I

Effect on the Anti-SRBC PFC Response of Adding 1 ng of TT on Day 2 to Mixed Cultures of 10, 1, or 0.1 Million TT-Primed Thymocytes and 10 Million Normal Spleen Cells Stimulated with SRBC on Day 0

Group	Cultured cells (in millions)	TT	Anti-SRBC plaques per 10^6 recovered cells				Average enhancement of anti-SRBC PFC response	P*
			Experiment					
			1	2	3	4		
1	10 Primed Thy 10 Normal Spl	+	250	1793	1530	400		
			(55)	(103)	(68)	(43)	67%	
2	10 Primed Thy 10 Normal Spl	−	161	883	913	280		
								<0.005
3	10 Normal Thy 10 Normal Spl	+	172	1623	1380	260		
			(−34)	(0)	(−15)	(−32)	−20%	
4	10 Normal Thy 10 Normal Spl	−	261	1617	1633	380		
5	1 Primed Thy 10 Normal Spl	+	1153	1527	973	1800		
			(75)	(153)	(37)	(91)	89%	
6	1 Primed Thy 10 Normal Spl	−	657	603	710	940		
								<0.005
7	1 Normal Thy 10 Normal Spl	+	810	1583	853	2260		
			(−18)	(−17)	(−33)	(−5)	−18%	
8	1 Normal Thy 10 Normal Spl	−	983	1910	1267	2390		
9	0.1 Primed Thy 10 Normal Spl	+	2027	1173	2060	1647		
			(−7)	(−4)	(−13)	(12)	−3%	
10	0.1 Primed Thy 10 Normal Spl	−	2173	1227	2367	1467		
11	0.1 Normal Thy 10 Normal Spl	+	2307	947	2567	1880		
			(−8)	(−34)	(−6)	(−19)	−17%	
12	0.1 Normal Thy 10 Normal Spl	−	2500	1433	2720	2307		
13	20 Primed Spl	+	1453	3347	2737	1043		
			(61)	(56)	(34)	(51)	51%	
14	20 Primed Spl	−	903	2140	2050	690		
								<0.001
15	20 Normal Spl	+	660	2587	2173	797		
			(−9)	(−3)	(−16)	(−8)	−9%	
16	20 Normal Spl	−	726	2660	2587	863		

Nos. in parentheses indicate degree of enhancement in per cent.

* Calculated by Student's t test to determine the significance of the difference between the mean enhancement produced in TT-primed cultures and the mean increase produced by the corresponding, similarly-treated, normal cultures.

TABLE II

Effect on the Anti-SRBC PFC Response of Adding 1 ng of TT on Day 2 to Mixed Cultures of 10 Million (or 1 Million) TT-Primed Spleen Cells and 10 Million Normal Spleen Cells Stimulated with SRBC on Day 0

Group	Cultured cells (in millions)	TT	Anti-SRBC plaques per 10^6 recovered cells				Average enhancement of anti-SRBC PFC response	*P*
			Experiment					
			1	2	3	4		
1	10 Primed Spl 10 Normal Spl	+	2620	2480	2553	2100		
			(61)	(89)	(61)	(133)	86%	
2	10 Primed Spl 10 Normal Spl	−	1627	1313	1587	903		
								<0.005
3	10 Normal Spl 10 Normal Spl	+	1547	1833	1880	2196		
			(−42)	(1)	(−13)	(4)	−12%	
4	10 Normal Spl 10 Normal Spl	−	2687	1813	2173	2103		
5	1 Primed Spl 10 Normal Spl	+	867	1333	2247	2040		
			(−18)	(−35)	(−15)	(10)	−14%	
6	1 Primed Spl 10 Normal Spl	−	1060	2053	2640	1853		
7	1 Normal Spl 10 Normal Spl	+	1040	1807	1473	1880		
			(−30)	(−3)	(−21)	(−8)	−15%	
8	1 Normal Spl 10 Normal Spl	−	1487	1867	1867	2053		
9	20 Primed Spl	+	1740	1840	2933	2827		
			(50)	(71)	(58)	(42)	55%	
10	20 Primed Spl	−	1160	1073	1853	1993		
								<0.001
11	20 Normal Spl	+	900	1380	1673	2067		
			(−20)	(−12)	(6)	(1)	−6%	
12	20 Normal Spl	−	1120	1567	1573	2047		

See Table I for explanatory footnotes.

1 ng of TT was added at day 0. Cell-free supernatants were harvested at daily intervals and were assayed for biological activity. Table III is a typical group of experiments which show the reproducible effect on the anti-SRBC PFC response of adding 24 hr supernatants on day 2 to normal spleen cell cultures that received SRBC on day 0. Addition of $ThyT^+$ supernatants derived from specifically-stimulated primed thymocytes to assay cultures resulted in a

TABLE III

Effect on the Anti-SRBC PFC Response of Adding 24-hr Thymocyte Culture Supernatants on Day 2 to Normal Spleen Cell Cultures That Received SRBC on Day 0

Supernatant fluid from cultured thymocytes from primed or normal animals			Assay culture of normal spleen cells to which supernatant was added			P*
			Anti-SRBC plaques per 10^6 recovered cells			
Exp.	Origin	Antigens added to culture	Final dilution of supernatant			
			1:10	1:30	1:100	
1	TT-primed	TT	1447 (−21)	1453 (−22)	2860 (+51)‡	
	Normal	TT	2112 (+15)	1480 (−20)	1873 (−1)	
	Normal	None	1840	1860	1893	
2	TT-primed	TT	1327 (+7)	1247 (−5)	1687 (+42)	
	Normal	TT	1200 (−3)	1200 (−8)	1353 (+14)	<0.005
	Normal	None	1240	1307	1190	
3	TT-primed	TT	1687 (−14)	2027 (+6)	3040 (+47)	
	Normal	TT	1887 (−4)	1873 (−2)	1907 (−8)	
	Normal	None	1973	1920	2073	

* Calculated by Student's t test to determine the significance of the difference between the mean enhancement produced by the optimum dilutions of supernatants from TT-primed cultures and the mean increase produced by the corresponding dilutions of normal supernatants.

‡ Numbers in parentheses indicate the degree of enhancement (in per cent) compared with the PFC response of normal cultures that had received supernatant from normal, unstimulated cultures.

significant increase in the anti-SRBC plaque response compared with similar cultures that received normal control supernatants. The average increase obtained, with a 1:100 dilution of the active supernatant, was 47% ($P < 0.005$). No such increase was found with culture medium from TT-stimulated normal thymocytes (Table III) or from unstimulated primed thymocytes (Fig. 1). Individual supernatants contained varying amounts of enhancing activity since optimal augmentation of the hemolytic plaque response was obtained with either 1:30 or 1:100 dilutions of the culture fluid (Tables III–VI). Dilutions other than the optimal dilution generally depressed the PFC response or enhanced it to an insignificant degree.

Kinetics of Release of Enhancing Factor.—Fig. 1 shows the results of a representative experiment in which supernatants from TT-primed thymocytes, maintained in the presence or absence of toxoid, were harvested from replicate cultures at 24-hr intervals and tested for enhancing activity. It can be seen that virtually all of the enhancing material was released from TT-stimulated primed thymocytes by 48 hr of culture, although a significant amount of activity was present in the 24-hr supernatants (Table III). The anti-SRBC PFC response

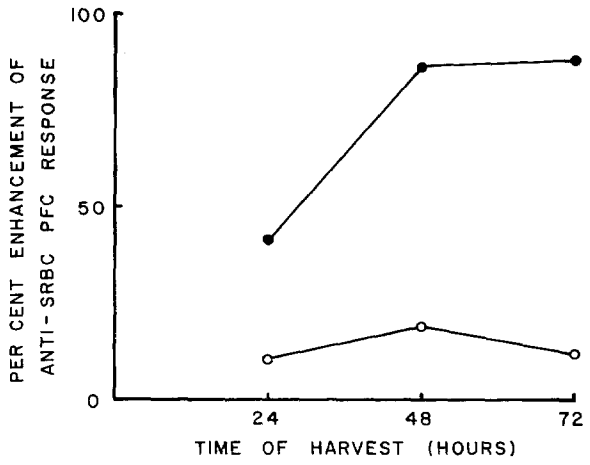

FIG. 1. Kinetics of release of enhancing factor from TT-primed thymocytes cultured with tetanus toxoid. Assay of culture supernatants from replicate cultures at 24, 48, and 72 hr. (●——●), $ThyT^+$ supernatants; (○——○), $ThyT^-$ supernatants.

was not significantly increased by culture fluid from TT-primed thymocytes maintained without TT (Fig. 1, lower curve).

Effect of Heat Treatment on Activity of Thymocyte-Derived Enhancing Factor.—Aliquots of $ThyT^+$ supernatants, harvested at 48 hr, were heated at 56°C for 30 min and tested for the ability to amplify the production of anti-SRBC plaques in assay cultures compared to the enhancing activity of similar, unheated aliquots. Table IV (experiments 1 and 2) demonstrates that the activity present in the $ThyT^+$ supernatants was unaffected by mild heat treatment. Diluted aliquots of heated and unheated supernatants produced comparable degrees of enhancement of the PFC response when added on day 2 to normal spleen cell cultures that received SRBC on day 0.

Effect of Dialysis on Activity of Thymocyte-Derived Enhancing Factor.—Supernatants from 48-hr cultures of TT-primed, TT-stimulated thymocytes were divided into aliquots. One aliquot was dialyzed against 1000 volumes of isotonic saline for 24 hr at 4°C, while the other was kept cold, but not dialyzed. The results from two independent experiments are shown in Table IV (experiments 3 and 4). It can be seen that the dialyzed and undialyzed supernatants were equally effective in amplifying the anti-SRBC PFC response of the assay cultures.

Effect of DNAse, RNAse, and Protease on Enhancing Factor.—Culture fluids from TT-stimulated spleen cells or thymocytes were divided and treated with one of three enzymes (DNAse I, RNAse A, or protease) in an attempt to define the chemical nature of enhancing factor. One aliquot of each supernatant was mixed with 10 μg/ml of enzyme for 1 hr at 37°C, while a similar aliquot was left untreated. Tables V and VI demonstrate that the activity of enhancing

TABLE IV

Effect on the Anti-SRBC PFC Response of Adding Heat-Treated or Dialyzed 48-hr Culture Supernatants on Day 2 to Normal Spleen Cell Cultures That Received SRBC on Day 0

Supernatant fluid from cultured thymocytes from primed or normal animals			Supernatant treatment	Assay culture of normal spleen cells to which supernatant was added		
				Anti-SRBC plaques per 10^6 recovered cells		
				Final dilution of supernatant		
Exp.	Origin	Antigens added to culture		1:10	1:30	1:100
1	TT-primed	TT	None	1720 (−19)	2573 (+43)	1940 (−2)
	TT-primed	TT	Heat*	2020 (−5)	2607 (+45)	2167 (+9)
	Normal	None		2127	1800	1980
2	TT-primed	TT	None	2087 (−1)	2360 (+28)	2760 (+64)
	TT-primed	TT	Heat*	2127 (+1)	2400 (+30)	2847 (+69)
	Normal	None		2100	1840	1687
3	TT-primed	TT	None	2047 (+1)	2593 (+50)	2133 (+9)
	TT-primed	TT	Dialysis‡	2433 (+20)	2520 (+46)	1887 (−4)
	Normal	None		2033	1727	1960
4	TT-primed	TT	None	1780 (+2)	1693 (−7)	2633 (+49)
	TT-primed	TT	Dialysis‡	1967 (+12)	2073 (+14)	2647 (+50)
	Normal	None		1753	1820	1767

See Table III for explanatory footnotes.

* Supernatants heated at 56°C, 30 min.

‡ Supernatants dialyzed against 1000 volumes of normal, isotonic saline for 24 hr at 4°C.

factor was not significantly affected by treatment with DNAse or RNAse, respectively, whether EF was derived from spleen or thymocyte culture medium. However, exposure of active supernatants to protease resulted in a significant decrease in their ability to augment the anti-SRBC response of normal spleen cells (Tables V and VI). As a control for possible toxicity of protease to the assay cultures, 1 μg of the enzyme, the maximum amount added with diluted supernatants to test cultures, was added to SRBC-stimulated spleen cell cultures. Similar numbers of plaques were obtained in cultures maintained with or without this amount of protease. Thus, the reduction of enhancing activity was due to enzymatic degradation of the factor, and not due to toxicity.

DISCUSSION

It is now clearly recognized that thymus-derived lymphocytes play an important functional role in facilitating the humoral immune response of B cells to a variety of antigens (10, 20, 21). But the actual mechanism of the

TABLE V

Effect on the Anti-SRBC PFC Response of Adding Enzyme-Treated, Spleen Cell Culture Supernatants on Day 2 to Normal Spleen Cell Cultures that Received SRBC on Day 0

Supernatant fluid from cultured spleen cells from primed or normal animals			Supernatant treatment*	Assay culture of normal spleen cells to which supernatant was added			P
				Anti-SRBC plaques per 10^6 recovered cells			
				Final dilution of supernatant			
Exp.	Origin	Antigens added to culture		1:10	1:30	1:100	
1	TT-primed	TT, SRBC	None	527 (−42)	733 (−40)	1147 (+41)	
	TT-primed	TT- SRBC	DNAse	853 (−6)	770 (−37)	1277 (+57)	
	Normal	None		910	1213	813	
2	TT-primed	TT, SRBC	None	1243 (−11)	1190 (−3)	1450 (+42)	
	TT-primed	TT, SRBC	DNAse	1490 (+7)	1470 (+19)	1450 (+42)	
	Normal	None		1397	1233	1020	
3	TT-primed	TT, SRBC	None	2413 (−1)	3460 (+46)	3673 (+62)	
	TT-primed	TT, SRBC	RNAse	2800 (+14)	3110 (+31)	3380 (+49)	
	Normal	None		2447	2373	2273	
4	TT-primed	TT	None	1753 (−28)	2540 (+9)	3200 (+50)	
	TT-primed	TT	RNAse	1827 (−25)	2493 (+7)	3180 (+49)	
	Normal	None		2427	2327	2140	
5	TT-primed	TT, SRBC	None	1560 (−40)	2967 (−17)	3500 (+40)	
	TT-primed	TT, SRBC	Protease	1407 (−46)	2247 (−10)	2367 (−5)	
	Normal	None		2620	2493	2500	
6	TT-primed	TT	None	1547 (−41)	2093 (−26)	3900 (+42)	
	TT-primed	TT	Protease	2920 (+11)	3047 (+7)	2653 (−3)	<0.010
	Normal	None		2627	2847	2740	
7	TT-primed	TT	None	1447 (−25)	1853 (−17)	2800 (+58)	
	TT-primed	TT	Protease	2060 (+7)	1753 (−22)	2013 (+14)	
	Normal	None		1927	2240	1773	

* Supernatants treated with 10 μg/ml of enzyme, 1 hr, 37°C. See Table III for explanatory footnotes.

"helper cell" activity is not yet understood. A number of alternative experimental models have been proposed to explain the mechanism of T cell–B cell interaction (22, 23). These hypotheses were summarized by Dutton et al. (23) as follows. (*a*) The T cell concentrates antigen on its surface, presumably through binding to specific receptor molecules, and presents multiple determinants to the B cell, forming an antigen-receptor bridge between the two lymphocytes. (*b*) Surface membrane interaction between T and B cells, in addition to antigen-bridging between the cells, is needed. (*c*) The B cell must receive two signals: one from its own receptor via antigen-binding and a second signal from the "carrier antibody" receptor on, or released by, the T cell. (*d*) Specifically activated T cells release a nonspecific chemical mediator which triggers B cells that have already bound antigen through receptors on their

TABLE VI

Effect on the Anti-SRBC PFC Response of Adding Enzyme-Treated, Thymocyte Culture Supernatants on Day 2 to Normal Spleen Cell Cultures That Received SRBC on Day 0

Supernatant fluid from cultured thymocytes from primed or normal animals			Supernatant treatment*	Assay culture of normal spleen cells to which supernatant was added			P
				Anti-SRBC plaques per 10^6 recovered cells			
				Final dilution of supernatant			
Exp.	Origin	Antigens added to culture		1:10	1:30	1:100	
1	TT-primed	TT	None	913 (−16)	940 (−15)	1373 (+45)	
	TT-primed	TT	DNAse	913 (−16)	913 (−17)	1307 (+38)	
	Normal	None		1087	1100	947	
2	TT-primed	TT	None	2607 (−10)	3040 (+9)	4200 (+48)	
	TT-primed	TT	DNAse	3020 (+4)	2833 (+1)	4000 (+41)	
	Normal	None		2900	2800	2840	
3	TT-primed	TT	None	1473 (−21)	1453 (−21)	2447 (+38)	
	TT-primed	TT	RNAse	1987 (+6)	1720 (−6)	2407 (+36)	
	Normal	None		1867	1833	1767	
4	TT-primed	TT	None	2310 (−18)	2910 (+9)	3930 (+46)	
	TT-primed	TT	RNAse	2560 (−10)	2647 (−1)	3870 (+43)	
	Normal	None		2833	2667	2700	
5	TT-primed	TT	None	1667 (−44)	3216 (+16)	4033 (+48)	
	TT-primed	TT	Protease	1487 (−50)	2107 (−24)	2753 (+1)	
	Normal	None		2993	2767	2727	
							<0.005
6	TT-primed	TT	None	2087 (+2)	2820 (+44)	2027 (−7)	
	TT-primed	TT	Protease	1360 (−34)	1980 (+1)	1860 (−15)	
	Normal	None		2053	1953	2180	

* Supernatants treated with 10 μg/ml of enzyme, 1 hr, 37°C. See Table III for explanatory footnotes.

surface membranes. The result of such stimulation of B cells is the production of immunoglobulin molecules which carry the same specificity as that of the receptor for antigen on the B cell surface. An accessory cell, possibly the macrophage, may also play an essential role in T cell–B cell interactions (22, 24, 25).

Our data strongly supports model *d*, although the other postulated mechanisms of collaboration between T and B lymphocytes cannot be totally excluded. We have demonstrated that an enhancing factor (EF) is specifically released from presumably sensitized lymphoid cells by interaction with priming antigen. Once elicited, it can augment the immune response to another, unrelated, antigen. The enhancing activity was generated in vitro by the addition

of the specific antigen used for priming to cultures of spleen cells or thymocytes derived from the immunized mice. A similar addition of priming antigen to cultures of normal cells, or cells from mice primed with a heterologous immunogen (15), failed to evoke the release of EF. The EF's found in the supernatant medium of cultures of either spleen cells, or thymocytes had similar physicochemical characteristics. The activity of the mediator was nondialyzable and unaffected by mild heat treatment (56°C, 30 min) or treatment with DNAse or RNAse. However, it was inactivated by exposure to protease. Although the factor was not destroyed by pancreatic RNAse or DNAse, this does not necessarily exclude the possibility that it can be inactivated by enzymes with other specificities or with RNAse A or DNAse I under different experimental conditions. We conclude, however, that the soluble factor is protein in nature.

The fact that an enhancing factor which can affect the productive response of B cells is released specifically from primed T cells diminishes the need to postulate actual antigen-bridging between the two classes of cells as a prior requirement for activation of the B cell component (models *a* and *b*). With a diffusable, soluble effector molecule, close proximity between the cells would be sufficient. Alternatively, the mediator that we have described could be a T cell receptor (or carrier antibody), possibly complexed with specific antigen. When bound to a receptor for antibody (26) or for antigen-antibody complexes (27) found on the B cell surface, it might induce antibody synthesis in those B cells that have also bound another antigen by specific antigen-receptor molecules. This double-hit hypothesis is a variation of model *c* described above. However, the fact that active supernatants were effective in augmenting the anti-SRBC PFC response when added to assay cultures on day 2, but not when added on day 0 of a 5-day culture period (15), may argue against this latter hypothesis. By day 3, substantial numbers of PFC are present in the spleen cell cultures (16). Thus, it is not inconceivable that most of the B cells which can be affected by EF are already past the stage of differentiation at which they could be stimulated sequentially by antigen and a complex. Moreover, cellular interactions, both in vitro (28–30) and in vivo (31, 32), between mixed populations of histoincompatible lymphoid cells produce nonspecific augmentation of immune responses similar to those we have described; indeed, it is likely that all these phenomena have a common mechanism which is associated with blast transformation. In fact, soluble factors produced by T cells in allogeneic cell mixtures have been reported (33, 34). Thus, if mere contact between allogeneic cells is sufficient to trigger an elevated, nonspecific response, it is unlikely that an antigen-antibody complex, or an anti-carrier antibody derived from T cells, is involved in these cell-to-cell interactions. Furthermore, a discrete, adjuvant-like molecule, produced under a variety of clinical and experimental conditions, could explain many of the nonspecific anamnestic responses reported over the years (13). Other possible mechanisms of action of EF have been discussed previously (15).

A number of nonantibody mediators of cellular immunity have been reported in recent years (35). These soluble factors, which mediate in vitro phenomena correlated with delayed hypersensitivity observed in vivo, nonspecifically amplify the activities of a small number of specifically stimulated lymphocytes. Effector molecules which apparently can regulate the humoral immune response have also been described (33, 34, 36–38). Feldmann and Basten (38) have demonstrated that carrier-primed T cells, stimulated with specific hapten-carrier complex, could collaborate with similarly stimulated B lymphocytes to produce a response to the hapten (DNP), although the lymphocytes were physically separated by a cell-impermeable membrane. B cells were unable to respond to the complex in the absence of carrier-stimulated T cells. However, under similar conditions, the factor released specifically from carrier-primed T cells did not augment the immune response to donkey erythrocytes. Therefore, this factor may differ from EF since it only selectively enhanced the response of B cells to specific determinants linked to the specificities recognized by T cells.

Two T cell-derived factors which restored the immune response of T cell-depleted spleen cells to SRBC have recently been described (36, 37). That found by Doria and his colleagues was released from thymocytes cultured with or without antigen (36). Another such material, reported by Gorczynski et al., was nondialyzable and was released by incubation with antigen (37). This latter material is very similar to the substance we have described. Schimpl and Wecker (33) and Britton (34) also found soluble factors which nonspecifically augmented the anti-SRBC PFC response but which were elicited by mixing T cells with lymphoid cells of a different H-2 type. These T cell-replacing factors and EF may have similar characteristics. However, the mediator of Britton (34) can only be found in the supernatants from 24-hr cultures of allogeneic cells, not in media from 48- to 120-hr cultures. Since EF persists for at least 72 hr (Fig. 1), there is a difference in the stability of the two effector molecules. Alternatively, inhibitory substances which mask the effect of stimulatory factors produced in allogeneic mixtures may also be present in the 48-hr to 120-hr supernatants. Thus, of the soluble factors recently described, only EF has been at least partially characterized with respect to physicochemical properties. Consequently, although they act similarly biologically, whether one or more of these mediators are identical awaits further experimentation.

In Table I we have shown that antigenic stimulation affects a relatively small number of cells in the thymus in such a way that they respond specifically to it again on restimulation. This response on the part of primed thymocytes was manifest by the nonspecific enhancement of the response of normal spleen cells to an unrelated antigen. Thus, the addition of TT to mixed cell cultures containing 10 million or 1 million, but not 0.1 million, TT-primed thymocytes resulted in the augmentation of the anti-SRBC hemolytic plaque response. These data support the concept that a portion of T cell memory resides in the immunocompetent cells of the thymus. In addition, tenfold fewer primed

thymocytes than primed spleen cells are needed to produce comparable degrees of enhancement. Specific stimulation of mixed cultures containing 1 million TT-primed thymocytes produced augmentation of the PFC response (Table I), whereas similar treatment of cultures containing 1 million primed spleen cells resulted in a slightly depressed plaque response (Table II). Mixed cultures with 10 million primed spleen cells, when stimulated with TT, showed enhancement (Table II). These data suggest that there may be more antigen-reactive cells in the thymus than in the spleen, or that T cells from the thymus, which are capable of responding to anamnestic stimulation in vitro, are more reactive than similar types of memory cells from the spleen. On the other hand, there may be no real difference in the number of EF-producing cells from these two lymphoid organs. In fact, with respect to rosette-forming, antigen-binding cells, tenfold more are found in the spleen than in the thymus (39). It is possible that the disparity in the number of spleen cells or thymocytes needed to produce comparable degrees of enhancement reflects the presence of a more balanced regulatory control mechanism in the spleen in the form of inhibitor-releasing, lymphoid cells which can antagonize the effects of EF-releasing components.

In a previous paper we reported that in mixed cell cultures containing 1 million thymocytes (or 10 million spleen cells) from TT-primed mice and 20 million normal spleen cells, no enhancement could be elicited (14). It is apparent from Tables I and II that when the priming antigen was added to cultures of 1 million primed thymocytes (or 10 million primed spleen cells) mixed with only 10 million normal spleen cells, half the number used before, enhancement of the anti-SRBC PFC response was observed. Thus, this number of primed thymocytes (or spleen cells) was reactive to anamnestic stimulation, but their activity was apparently masked by some component provided by the 20 million normal spleen cells used in the earlier experiments. This masking phenomenon may be due to some type of soluble inhibitory molecule. The presence of such an inhibitor in the supernatants of specifically stimulated lymphoid cell cultures has been reported (15, 40).

In conclusion, specific antigenic stimulation of thymocytes from primed animals results in the release of a soluble factor which can nonspecifically amplify the humoral immune response of normal spleen cells that are responding to a heterologous, non-cross-reacting antigen. A factor with similar physicochemical properties is also released in vitro by anamnestic stimulation of primed spleen cells. The mediator is most likely protein in nature. An effector molecule such as the one we have described may mediate cellular interactions between T cells and B cells in the induction of antibody synthesis.

SUMMARY

In short-term cultures of thymocytes from tetanus toxoid-immunized mice, the addition of 1 ng of toxoid generated the release of a soluble factor which was capable of enhancing the immune response to a heterologous immunogen. The

addition of supernatants from such cultures to assay cultures of sheep erythrocyte-stimulated normal spleen cells produced a significant augmentation of the hemolytic plaque response. Culture fluid from similar cultures of normal thymocytes or primed thymocytes cultured without the priming antigen were inactive. The enhancing factor was nondialyzable, heat stable (56°C, 30 min), resistant to DNAse and RNAse, but was inactivated by protease. A factor produced by specifically stimulated primed spleen cells had similar characteristics. In toxoid-stimulated, mixed cell cultures containing primed thymocytes or spleen cells and normal spleen cells, tenfold fewer thymocytes than spleen cells were needed to produce a comparable degree of enhancement of the anti-sheep erythrocyte plaque-forming cell response.

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