SYNERGY BETWEEN SUBPOPULATIONS OF MOUSE SPLEEN CELLS IN THE IN VITRO GENERATION OF CELL-MEDIATED CYTOTOXICITY

Evidence for the Involvement of a Non-T Cell

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The generation of allograft-like cell-mediated cytotoxicity during the in vitro mixed lymphocyte culture (MLC)[1] has been well described (1, 2). The cytotoxic effector cell so generated has been characterized as a thymus-dependent (T) lymphocyte by several investigators (review-3). In addition, a role of macrophages has been proposed during both sensitization (4) and effector (5) phases. More recently, synergy between two lymphoid populations has been described in this system. Wagner (6), Cohen and Howe (7), Häyry and Andersson (8), and Tigelaar and Feldmann (9) have described synergy between mouse thymus and peripheral lymphocytes in an in vitro allograft system. In all instances, synergy was demonstrated between cell populations derived from two different lymphoid organs, and in each report this synergy was interpreted as involving two thymus-dependent populations. The present study also demonstrates synergy, but described interaction between two subpopulations separated from the same peripheral lymphoid organ, mouse spleen. In addition, evidence is presented that the activity of one of these subpopulations appears to be distinct from effector cell activity, does not possess some T-cell characteristics, and could not be replaced by macrophage preparations.

Materials and Methods

Aniamls. Mice were of the inbred strains C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) maintained at the National Institutes of Health, Bethesda, Md. All animals used were male, aged 8-12 wk.

Cell Preparations. Sterile spleen cell suspensions were prepared and red cell lysed as previously described in detail (10).

In experiments designed to examine the possible role of spleen macrophages in the system studied a plastic-adherent spleen cell population was used (Dr. D. Mosier, NIH, personal communication). Fixed numbers of spleen cells were portioned in 1 ml of serum-free Eagle's minimal essential medium

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[1] *Abbreviations used in this paper:* MEM-FCS, MEM containing 10% fetal calf serum; MLC, mixed lyphocyte culture; PEC, peritoneal exudate cells, RαMB, rabbit antimouse brain serum.

(MEM) (NIH media unit) in each flat-bottom well of plastic multiwell dishes (flat-bottom 16 mm diameter; FB-16-24-TC; Linbro Chemical Co., Inc., New Haven, Conn.). After 5 days of incubation at 37°C in an air-7% CO_2 atmosphere, the wells were washed by repeated pipetting, leaving a residual population adhering to the bottom of each well. These wells were then used as a macrophage-rich environment for in vitro sensitizations (vide infra).

Peritoneal exudate cells (PEC) were prepared from mice injected intraperitoneally 72 h earlier with 1 ml of sterile 10% proteose-peptone broth (Difco, Detroit, Mich.). Heparinized (10 U/ml) magnesium- and calcium-free Spinner's medium at 4°C was used to irrigate the peritoneal cavity; cells were then washed in heparin-free Spinner's medium at 4°C, resuspended in MEM, counted, and added immediately to in vitro sensitization cultures.

Cell Separation. Spleen cells were fractionated by nylon wool column passage, with minimal modification of the method described by Julius et al. (11) and by Handwerger and Schwartz (12). 1.2 g of nylon wool (FT-242; Fenwal Laboratories, Morton Grove, Ill.) which had been prewashed in glass-distilled water was packed into the barrel of a 20 ml disposable plastic syringe (Sherwood Medical Industries, Deland, Fla.) to the 12 ml mark and autoclaved. Before cell application, the nylon in the column was saturated and rinsed with MEM containing 10% fetal calf serum (MEM-FCS), and the column was incubated for 1 h at 37°C. After this preincubation, 300×10^6 spleen cells in 4 ml of MEM-FCS, preheated to 37°C, were added to the column and allowed to flow into the nylon wool followed by 2 ml of 37°C MEM-FCS. The column was then incubated for 45 min in a 37°C humidified air-7% CO_2 incubator. After this incubation, nonadherent cells were removed by adding MEM-FCS at 37°C to the column, and adjusting outflow with a three-way stopcock to approximately 1 ml/min until 15 ml of effluent was collected. The column was then washed with 100 ml of 37°C MEM-FCS flowing rapidly through the column and this wash was discarded. Column-adherent cells were removed by compressing the nylon wool with the syringe plunger to express retained medium, removing the plunger, teasing the nylon up with sterile forceps, resaturating with medium, and compressing again, collecting approximately 50 ml in five or six cycles of compression.

Cell Markers. Cell surface immunoglobulin (Ig) as a B-cell marker was identified by fluorescein-conjugated rabbit antimouse immunoglobulin (Cappel Laboratories, Downington, Pa., lot no. 6117) as described by Dickler and Sachs (13). Rabbit antimouse brain serum (RαMB) (vide infra) was used as a T-cell marker by indirect fluorescence, employing a rhodamine-conjugated goat antirabbit immunoglobulin (Cappel, lot no. 6638). Phagocytes were identified by their ability to ingest latex beads (14), incubated in the presence of 20% FCS.

Rabbit Antimouse Brain Serum. RαMB was prepared by modification of the method of Golub (15), by subcutaneous innoculation of homogenate of one BALB/c mouse brain in equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) per rabbit, boosting 1 wk later with an identical dose and preparation, and bleeding 1 wk after this challenge. After heat inactivation (30 min at 56°C), serum was absorbed with BALB/c peripheral blood cells, 0.4 ml packed cells per ml serum. Plateau titer measured by ^{51}chromium (^{51}Cr)-release cytotoxicity against BALB/c spleen cells was $\geq$1:256, against lymph node cells 1:1,000, and against thymus 1:4,000. By indirect immunofluorescence, the reagent diluted 1:60 stained 34% of spleen cells, 57% of mesenteric lymph node cells, and 98% of thymus cells. When RαMB was used as a preparative reagent, cells were suspended at a concentration of 5×10^6/ml in RαMB diluted 1:60 in MEM-FCS, incubated 30 min at room temperature, then centrifuged, washed once, and incubated at 10^7/ml for 30 min at 37°C in guinea pig complement (lyophilized guinea pig complement, Grand Island Biological Co., Grand Island, N. Y., lot no. A232522) which had been absorbed with BALB/c spleen cells (one spleen per ml of undiluted complement), and was then used at a dilution of 1:3. They were then washed three times with MEM-FCS, resuspended and viable cells counted. Complement controls were treated identically except that MEM-FCS was substituted for RαMB during incubation.

In Vitro Sensitization. In vitro sensitization was carried out by mixed cell culture under conditions modified by Wunderlich (personal communication) from those described by Mishell and Dutton (16). Incubations were carried out in MEM supplemented as previously described (16), with the further addition of 2-mercaptoethanol at a final concentration of 5×10^{-5} M. Nonirradiated responding cells were mixed with 2,000 rad irradiated (specific conditions of irradiation previously described) (10) sensitizing cells in a total vol of 1 ml in each well of plastic multiwell dishes (FB-16-24-TC; Linbro Chemical Co., Inc.). The cells were incubated at 37°C in a humidified air-7%

CO_2 atmosphere for 5 days. During incubation, each well was "fed" daily with 0.2 ml of a nutrient medium (16). At the end of incubation, cells were harvested from each well by repeated pipetting and washing; replicate wells were pooled at this stage, cells centrifuged, resuspended in MEM-FCS and viable cells counted. These cells were used as effector (attacker) cells in cytotoxicity assay.

Cytotoxicity Assay. Target cells used were in vivo ascites passage tumor lines EL-4 ($H\text{-}2^b$) and LSTRA ($H\text{-}2^d$). These were ^{51}Cr-labelled by incubation of 20-50 × 10^6 cells/ml in MEM-FCS and 0.1 mCi/ml ^{51}Cr (as sodium chromate, Amersham Searle, Arlington Heights, Ill., approx. 1 mCi/mg Cr) for 30 min at 37°C, followed by three washes in MEM-FCS. 10^5 target cells and a number of effector cells giving an appropriate effector:target cell ratio were mixed in a total vol of 1 ml of MEM-FCS in 12 × 75 mm round-bottom plastic tubes (no. 2052, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) which were centrifuged for 2 min at 25 *g*, and then incubated for 4 h at 37°C in humidified air 7% CO_2. All tests were carried out in triplicate. Target cells were incubated alone to measure "spontaneous" release of isotope. Total counts incorporated in target cells were determined by direct counting of 10^5 labeled cells. At the conclusion of the 4 h, 1 ml of MEM-FCS at 4°C was added to each tube. After centrifuging the tubes for 10 min at 4°C, 700 *g*, 1 ml of supernate was removed from each tube and transferred to a 16 × 125 mm plastic tube (Amersham Searle) for counting on a well-type gamma counter (Nuclear Chicago). Cytotoxicity was expressed as cytotoxic index =

$$\frac{\text{(counts released from experimental group)-(counts from spontaneous release)}}{\text{(total counts of labeled target)-(counts from spontaneous release)}} \times 100$$

with mean ± standard error of the mean (SEM) for replicates presented.

Assays of Proliferation. Assays of tritiated thymidine incorporation and response to mitogens were performed by a microtiter assay which has already been described in detail (10).

Results

Cell Fractionations. In five separations in which cell surface Ig, RαMB immunofluorescence, and latex ingestion were quantitated, unseparated spleen cells were 43.2 ± 2.2% (mean ± SEM) Ig positive, 35.6 ± 1.0% RαMB positive, and 4.5 ± 0.6% latex-ingesting; column-nonadherent cells were 2.5 ± 0.6% Ig positive, 78.8 ± 1.9% RαMB positive, and 0.1 ± 0.1% latex-ingesting; and adherent cells 81.6 ± 1.6% Ig positive, 7.5 ± 0.9% RαMB positive, and 1.5 ± 0.5% latex-ingesting. By these criteria it is apparent that the nonadherent population is enriched for T cells and depleted of B cells; the adherent is enriched for B and depleted of T cells. Both column-passed populations are depleted of latex-ingesting cells, the depletion in the column-nonadherent population being more marked. The PHA and LPS responses of these separated cells paralleled the enrichment and/or depletion of T and B cells respectively. The total cell recovery from nylon column separations was 70 ± 4%, with higher recovery of Ig-bearing (93 ± 6%) than of RαMB positive cells (60 ± 3%), and a much lower recovery of latex-ingesting cells (14 ± 4%).

Specificity of In Vitro Sensitization. The specificity of the cytotoxicity induced during in vitro MLC has previously been described (17). It has been confirmed in the current experimental system by demonstrating that cells sensitized to a specific allogeneic cell type are not cytotoxic to syngeneic target cells or to other allogeneic cells with low serologic cross-reactivity. In addition, no "innocent bystander" killing was detected.

Generation of Cytotoxicity in Nylon-Separated Spleen Cell Populations. In order to assess the effects of responder cell density on in vitro sensitization, the

number of responding cells per culture was titrated for unseparated and nylon column-fractionated spleen cells, with the number of irradiated sensitizing cells held at 0.5×10^6 per culture (Table I). The relationship of cytotoxic potential generated to the number of responding cells/culture differed for each cell population. Unseparated cells were optimally sensitized at 1×10^6 cells/culture, column-adherent cells optimally at 4×10^6/culture (confirmed in additional titrations including higher numbers of cells/culture), and nonadherent cells on a plateau at 1×10^6–4×10^6 cells/culture. Cytotoxicity was assessed at multiple effector:target cell ratios, and comparisons of different effector populations were made under conditions at which the relationship of cytotoxic index to the number of effector cells was linear. In this instance, sensitized nonadherent cells were more cytotoxic than sensitized adherent cells at any culture condition examined.

TABLE I

Effect of Responder Cell Number on In Vitro Sensitization

Sensitization culture		Cytotoxic index (%) Target cells							
		EL-4				LSTRA			
Responder	Sensitizer*	4:1‡	2:1	1:1	1:2	4:1	2:1	1:1	1:2
4×10^6 BALB/c unseparated	0.5×10^6 C57BL/6	20.6	11.2	4.8	2.2	0.2			
4×10^6 BALB/c adherent	0.5×10^6 C57BL/6	49.6	31.3	19.2	9.7	1.3			
4×10^6 BALB/c nonadherent	0.5×10^6 C57BL/6	59.3	45.8	32.1	17.9	1.3			
2×10^6 BALB/c unseparated	0.5×10^6 C57BL/6	38.2	20.3	10.3	6.0	1.9			
2×10^6 BALB/c adherent	0.5×10^6 C57BL/6	41.1	22.6	11.7	7.0	0.4			
2×10^6 BALB/c nonadherent	0.5×10^6 C57BL/6	60.9	47.5	33.3	19.3	1.2			
1×10^6 BALB/c unseparated	0.5×10^6 C57BL/6	47.3	33.7	18.2	10.9	1.4			
1×10^6 BALB/c adherent	0.5×10^6 C57BL/6	16.6	9.8	4.9	2.9	0.5			
1×10^6 BALB/c nonadherent	0.5×10^6 C57BL/6	58.3	47.9	31.3	17.2	0.8			
0.5×10^6 BALB/c unseparated	0.5×10^6 C57BL/6	36.6	24.8	12.1	6.4	-0.4			
0.5×10^6 BALB/c adherent	0.5×10^6 C57BL/6	—	1.2	1.2	0.7	—	-0.3		
0.5×10^6 BALB/c nonadherent	0.5×10^6 C57BL/6	51.6	34.1	22.5	11.8	-0.4			
4×10^6 C57BL/6 unseparated	0.5×10^6 BALB/c	0.4	—	—	—	18.7	10.5	5.5	2.5

* Sensitizing cells were irradiated unseparated spleen cells.

‡ Effector: target cell ratio.

Over multiple experiments, however, this relationship was variable. The sensitized nonadherent fraction was usually but not always more cytotoxic than unseparated cells; the cytotoxicity of the adherent fraction was less than or greater than that of the unseparated cells with equal frequencies. Therefore, none of these comparisons between individual populations allowed statement of a consistent relationship. The source of these variations in relationships could not be satisfactorily identified.

The Nature of Effector Cell Activity. Treatment with RαMB and complement increased the percentage of mouse Ig-bearing cells in unseparated spleen from 43% to 77%, and decreased the percentage of cells reacting with RαMB by indirect fluorescence from 35% to 0%. Treatment of the nylon column-frac-

tionated or unseparated spleen cells totally eliminated PHA responsiveness while increasing LPS responsiveness. These data support the T-cell specificity of RαMB.

The sensitivity to RαMB of the effector cell activity generated in unseparated spleen or its separated fractions was examined (Table II). In all three cell populations there was a complete abrogation of the cytotoxic effect of sensitized cells by treatment with RαMB and complement after sensitization, suggesting that the effector activity of all sensitized populations examined requires T-cell function.

TABLE II

Effect of Treatment with RαMB and Complement on Effector Cells Generated by In Vitro Sensitization

Sensitization culture*	Cytotoxic index (%) Target cell: EL-4		
	4:1‡	2:1	1:1
BALB/c unseparated – medium control	63.3 ± 1.0	46.9 ± 0.6	28.0 ± 0.6
BALB/c unseparated – complement control	69.1 ± 2.2	56.4 ± 1.8	34.9 ± 2.1
BALB/c unseparated – RαMB + complement treated	0.9 ± 0.4	1.4 ± 0.5	0.7 ± 0.3
BALB/c adherent – medium control	32.6 ± 0.7	17.1 ± 0.5	9.9 ± 0.6
BALB/c adherent – complement control	41.1 ± 0.8	23.8 ± 1.4	12.0 ± 0.6
BALB/c adherent – RαMB + complement treated	0.3 ± 0.5	1.0 ± 0.3	0.6 ± 0.3
BALB/c nonadherent – medium control	63.2 ± 1.4	45.6 ± 1.0	26.5 ± 1.2
BALB/c nonadherent – complement control	64.1 ± 1.9	46.6 ± 0.5	27.6 ± 0.6
BALB/c nonadherent – RαMB + complement treated	—	1.6 ± 0.4	0.8 ± 0.4

* Each culture consisted of 4×10^6 of the responders as shown, cultured with 0.5×10^6 irradiated unseparated C57BL/6 spleen cells.

‡ Effector: target cell ratio.

Synergy of Nylon Column Adherent and Nonadherent Spleen Fractions in In Vitro Sensitization. Sensitization was carried out in which the total number of responding cells/culture was held constant, and its composition varied by mixing adherent and nonadherent cells in several proportions. Fig. 1 *a* represents an experiment in which 4×10^6 C57BL/6 cells/culture were used as responders and 0.5×10^6 irradiated BALB/c spleen cells [$(\text{BALB/c})_x$] as sensitizers. It is apparent that mixtures of the two spleen cell fractions generated cytotoxicity greater than that which would be expected if there were no synergy between the two fractions, i.e., greater than the simple average of cytotoxicities generated in adherent cells alone and nonadherent cells alone.

Fig. 2 represents a similar experiment in which 4×10^6 BALB/c cells/culture are sensitized to 0.5×10^6 $(\text{C57BL/6})_x$/culture. Cytotoxic index is plotted as a

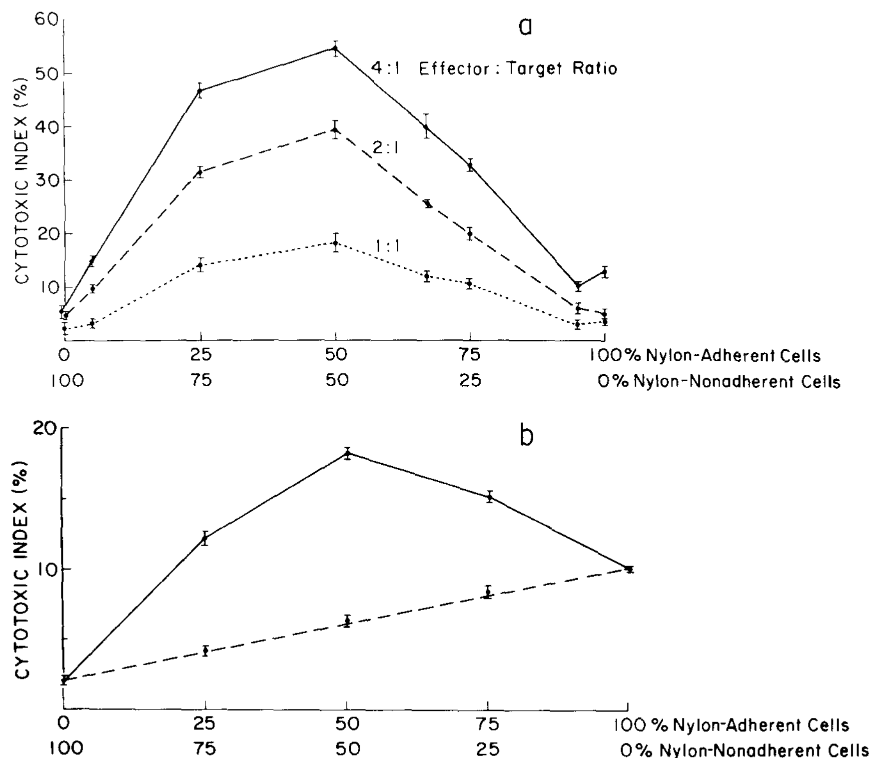

FIG. 1. Synergy during in vitro sensitization. The cytotoxic index is plotted for multiple effector populations indicated. (*a*) Effector cells were generated in mixtures of nylon column-adherent and nonadherent C57BL/6 spleen cells. 4×10^6 C57BL/6 responding cells and 0.5×10^6 irradiated BALB/c sensitizing cells were cultured in each sensitization culture. The composition of the responding cell mixture of adherent and nonadherent cells is indicated on the horizontal axis. (*b*) Points connected by the solid line represent effector cells generated by in vitro culture of 4×10^6 BALB/c responding cells in the proportion of nylon-adherent and nonadherent indicated on the horizontal axis. Points connected by the dotted line represent effector cells prepared by sensitizing 100% adherent or 100% nonadherent cells as responders, and after sensitization mixing these sensitized cells in the proportion indicated on the horizontal axis. During sensitization each responder population was cultured with 0.5×10^6 C57BL/6 irradiated unseparated sensitizing cells. Effector:targer ratio was 1:1.

function of the effector-target cell ratio for each sensitized responder population. An "expected" line which presumes no interaction between the fractions has been calculated for a 1:1 mix of adherent and nonadherent cells (2×10^6 adherent + 2×10^6 nonadherent per culture). The observed cytotoxicity generated in the 1:1 mixture is significantly greater than that "expected", demonstrating synergy between the two spleen cell populations. This relationship was seen consistently in each of 40 experiments performed; in each of these experiments, the cytotoxicity generated in the mixture was also greater than that generated in unseparated spleen.

Mechanism of Synergy. In order to distinguish synergy occurring during the sensitization phase from that occurring during the effector (cytotoxic) phase, spleen fractions were sensitized alone, and the independently sensitized cells

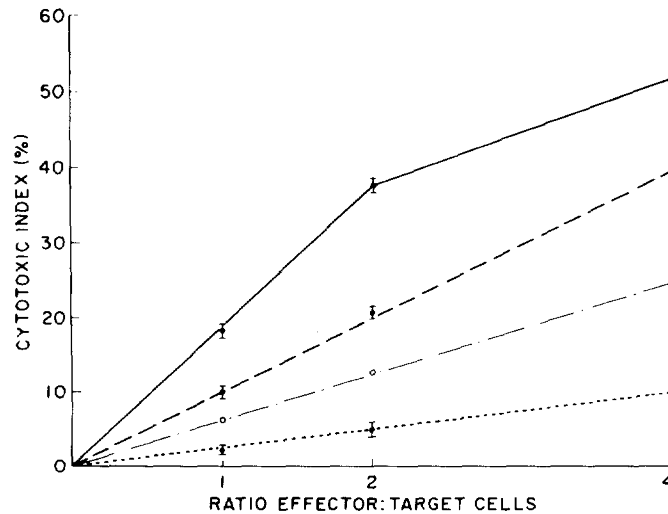

FIG. 2. Cytotoxicity generated in nylon column-separated BALB/c spleen cells. 4×10^6 BALB/c responding cells and 0.5×10^6 irradiated C57BL/6 unseparated sensitizing cells were cultured in each sensitization culture. Cytotoxicity at multiple effector:target cell ratios is plotted for each responder cell population. (----), 4×10^6 column-adherent cells; (- - -), 4×10^6 column-nonadherent cells; (——), 2×10^6 column-adherent + 2×10^6 column-adherent cells; (— · —), "expected" cytotoxicity for the mixture of adherent and nonadherent cells, calculated as the arithmetic mean of cytotoxicities generated by each population sensitized alone.

were then mixed as effector cells in cytotoxicity assay (Fig. 1 *b*). Mixtures of sensitized adherent and nonadherent cells were intermediate between the two in their cytotoxicity, equalling the activity predicted from the product of two noninteracting populations. In the same experiment, adherent and nonadherent cells mixed before sensitization showed synergy similar to that shown in Fig. 1 *a*. The observed synergy of spleen fractions thus appears to occur only during sensitization and not at the effector level.

Nature of Synergizing Cells. The nylon column-adherent population, although enriched for B lymphocytes, contained contaminating T cells. Investigation was therefore undertaken to examine the effect of treatment with RαMB and complement before sensitization upon the ability of nylon-adherent cells to generate cytotoxic activity. Table III illustrates the complete abrogation of the induction of cytotoxicity in adherent cells; treatment with complement in the absence of RαMB was without effect. Moreover, the RαMB-treated adherent cells, which had no measurable ability to generate cytotoxicity themselves, remained potent in their ability to synergize with 2×10^6 BALB/c nonadherent cells. The fact that this synergy was demonstrated at a range of cell densities (2-4 $\times$ 10^6 cells/culture) over which the cytotoxicity generated in the nonadherent cells alone was independent of density (Table I) (and where RαMB-treated adherent cells alone had no measurable cytotoxicity) argues against the possibility that the synergy observed is an artifact produced by differences in cell density in culture. An alternate means of expressing the cytotoxicity generated in responding populations is as cytotoxicity per culture, rather than per fixed

number of recovered cells. Table IV compares the total cytotoxicity generated per culture for nonadherent cells alone and for mixtures of nonadherent and RαMB-treated adherent cells. A "lytic unit" is here defined as the number of effector cells required to produce a cytotoxic index of 10%. The synergy between nonadherent and treated adherent cells is evident by this comparison as well.

The percent cell survival during 5-day sensitization culture (with allogeneic irradiated sensitizing cells) was frequently higher for mixtures of adherent and nonadherent cells than for either of these fractions sensitized alone (e.g. Table IV). When these responder populations were cultured without allogeneic sensitizers (but with syngeneic irradiated cells), no such survival advantage was observed in culture. This suggests that cell survival differences in culture are dependent upon allogeneic stimulation and do not represent less specific effects of in vitro conditions.

The ability of RαMB-treated adherent cells to synergize in in vitro sensitization suggests that the synergizing cell is a non-PHA responding, RαMB-negative (by fluorescence and cytotoxic susceptibility) cell distinct from the RαMB-sensitive T cell which is essential for cytotoxic effector activity in this system. These characteristics provide evidence supporting a non-T identity of this synergizing population.

Role of Macrophages. Since macrophages are among the non-T cells which

TABLE III

Effect of Treatment with RαMB and Complement on Synergy and on Direct Cytotoxic Ability of Column-Adherent Cells

Sensitization culture*	Cytotoxic index ± SEM (%) Target cell: EL-4			
	2:1‡	1:1	1:2	1:4
4×10^6 BALB/c adherent	26.4 ± 0.3	13.8 ± 0.5	7.6 ± 0.3	3.8 ± 0.1
4×10^6 BALB/c adherent RαMB + C′ treated	−0.4 ± 0.1	0.0 ± 0.2	0.3 ± 0.2	0.5 ± 0.2
2×10^6 BALB/c adherent	11.2 ± 0.5	5.9 ± 0.3	2.6 ± 0.2	1.4 ± 0.1
2×10^6 BALB/c adherent RαMB + C′ treated	0.0 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	—
4×10^6 BALB/c nonadherent	14.7 ± 0.2	9.6 ± 0.6	4.8 ± 0.3	2.9 ± 0.2
2×10^6 BALB/c nonadherent	12.6 ± 0.4	7.4 ± 0.2	3.2 ± 0.2	1.5 ± 0.1
2×10^6 BALB/c nonadherent + 0.1×10^6 BALB/c adherent, RαMB + C′ treated	17.5 ± 1.0	8.9 ± 0.1	4.4 ± 0.2	2.7 ± 0.2
2×10^6 BALB/c nonadherent + 0.5×10^6 BALB/c adherent, RαMB + C′ treated	18.9 ± 0.1	9.5 ± 0.5	4.5 ± 0.2	3.0 ± 0.2
2×10^6 BALB/c nonadherent + 1.0×10^6 BALB/c adherent, RαMB + C′ treated	32.9 ± 0.5	15.1 ± 0.5	7.8 ± 0.3	4.1 ± 0.2
2×10^6 BALB/c nonadherent + 2.0×10^6 BALB/c adherent, RαMB + C′ treated	44.4 ± 0.4	27.0 ± 1.1	13.4 ± 0.4	7.3 ± 0.2

* See Table II.

‡ Effector: target cell ratio.

TABLE IV
Synergy of Nylon Column Nonadherent and RαMB-Treated Adherent Cells in the Generation of Cytotoxicity

Sensitization culture*	Cell recovery per culture ($\times 10^{-5}$)	% Cell recovery	Cells per lytic unit‡ ($\times 10^{-5}$)	Lytic units per culture
2×10^6 BALB/c adherent cells, RαMB + C′ treated	3.4	17	—	0
2×10^6 BALB/c nonadherent cells	7.8	39	1.6	4.9
2×10^6 BALB/c nonadherent cells + 0.1×10^6 adherent, RαMB + C′ treated	8.0	38	1.15	7.0
2×10^6 BALB/c nonadherent cells + 0.5×10^6 adherent, RαMB + C′ treated	17.0	68	1.06	16.0
2×10^6 BALB/c nonadherent cells + 1.0×10^6 adherent, RαMB + C′ treated	17.4	58	0.64	27.2
2×10^6 BALB/c nonadherent cells + 2.0×10^6 adherent, RαMB + C′ treated	22.0	55	0.38	58.7

* See Table II.
‡ A lytic unit is defined as the number of effector cells required to lyse 10% (cytotoxic index) of 10^5 target cells.

may be active in this system of in vitro sensitization, several approaches were taken to examine their importance in the synergy which has been described.

The effect of adding PEC containing 58% latex-ingesting cells to sensitizing MLC's is seen in Table V. To 4×10^6 responding cells were added from 5×10^2 to 1×10^4 PEC; in experiments not shown greater numbers of PEC up to 8×10^4 were increasingly suppressive to nonadherent cells and to mixtures. There was no facilitation of cytotoxicity in nonadherent cells by the addition of this macrophage-rich preparation; i.e., nylon column-nonadherent cells, which were able to synergize with column-adherent cells, did not synergize with PEC. RαMB-treated adherent cells were without activity when cultured alone or with any number of PEC added. In contrast, small numbers of PEC (5×10^2/culture) did consistently increase the cytotoxicity generated in the mixtures of nonadherent and RαMB-treated adherent cells.

Since synergy was observed between spleen cell fractions, and splenic macrophages may be different from PEC, the effect of splenic macrophages was also investigated. Titrated numbers of spleen cells were preplated in culture wells in serum-free medium for 5 days; and the wells, with adhering cells enriched for splenic macrophages, were then washed and used as a macrophage-enriched environment for in vitro sensitizations. Although it was not possible to quantitate the macrophage dose per culture by this method, results were qualitatively similar to those obtained with PEC, showing suppression of the activity of all populations cultured in wells with higher numbers of preplated spleen cells and no significant effect of lower cell numbers.

As an additional approach, attempts were made to macrophage-deplete

TABLE V
Effect of Peritoneal Exudate Cells on In Vitro Generation of Cytotoxicity

Sensitization culture*	Cytotoxic index (%) Target cell				
	EL-4				LSTRA
	4:1‡	2:1	1:1	1:2	4:1
4×10^6 BALB/c nonadherent	66.6	57.4	43.0	23.8	1.0
4×10^6 BALB/c nonadherent + 1×10^4 PEC	55.6	50.8	27.3	16.9	0.8
4×10^6 BALB/c nonadherent + 5×10^3 PEC	61.3	49.7	27.5	15.3	0.4
4×10^6 BALB/c nonadherent + 5×10^2 PEC	60.2	42.8	27.1	15.7	0.7
4×10^6 BALB/c adherent, RαMB + C′ treated	−0.1	0.1	0.6	0.7	−0.7
4×10^6 BALB/c adherent, RαMB + C′ treated + 1×10^4 PEC	−0.1	0.1	0.5	0.2	0.0
4×10^6 BALB/c adherent, RαMB + C′ treated + 5×10^3 PEC	−0.2	0.1	0.1	1.0	−0.6
4×10^6 BALB/c adherent, RαMB + C′ treated + 5×10^2 PEC	−0.2	0.2	0.2	0.7	−0.4
4×10^6 BALB/c mix§	60.0	50.0	33.5	17.7	1.1
4×10^6 BALB/c mix + 1×10^4 PEC	63.7	53.2	33.1	17.3	2.0
4×10^6 BALB/c mix + 5×10^3 PEC	72.0	52.0	31.7	16.0	2.2
4×10^6 BALB/c mix + 5×10^2 PEC	75.9	62.0	43.5	25.4	2.8

* See Table II.
‡ Effector: target cell ratio.
§ Mix: a mixture of equal numbers of nonadherent cells and RαMB + C′-treated adherent cells.

populations of unseparated and nylon-separated spleen cells. G-10 Sephadex column passage (18), glass wool passage (11), and adherence to plastic (19) have been previously described as methods for depletion of macrophages. Each of these methods, while depleting of latex-ingesting cells, failed to eliminate the ability of nylon-adherent cells to synergize with nonadherent cells, thus providing further suggestion that the synergizing cell is not a macrophage. However, low total cell recoveries (40-70%) by the above methods complicate interpretation of the results.

Discussion

The present studies demonstrate a strong synergy between two subpopulations of spleen cells, separated by adherence or nonadherence to nylon wool, in the generation of cytotoxic effector cells during in vitro MLC. Experiments testing the effects of mixing these two cell populations before or after sensitization suggest that synergy occurs during sensitization and not at the effector level of cytotoxicity.

Initial efforts have been made to characterize the synergizing cells in this

system. The nylon column-adherent and nonadherent spleen fractions, respectively enriched for B cells and T cells, were not pure fractions. Since previous descriptions of synergy in in vitro sensitization have demonstrated an interaction of two T-cell subclasses, the effect of RαMB on synergy by the adherent fraction was investigated, in an effort to decrease its residual T-cell contamination. Although the cytotoxic or effector activity induced in either fraction was sensitive to RαMB treatment, consistent with the requirement for a T-cell at the effector stage, the ability of the adherent population to synergize during sensitization was not impaired by treatment with RαMB. Therefore, it must be concluded that at least one of the cells synergizing in sensitization is different both from cytotoxic effector (T) cells and PHA responsive (T) cells, and thus may be a non-thymus-dependent cell. Preliminary experiments employing treatment with an allogeneic anti-θ serum have produced results which are similar to those obtained with heterologous RαMB, and support these conclusions.

There is agreement by several authors that the macrophage plays an important role in the proliferative response of MLC (20) as well as in the in vitro generation of cytotoxic effector cells (4). Since the nylon wool column separation employed did alter macrophage content, it appeared possible that this non-T cell was participating in the synergy observed. However, the addition of peritoneal or splenic macrophages failed to produce any consistent effect other than a suppressive effect of large cell numbers, and a possible enhancement of the synergy of nylon-adherent and nonadherent cells by small numbers of PEC. Neither plastic-adherent spleen cells nor PEC were able to replace the synergizing function of nylon wool column-adherent cells. Several procedures designed to selectively deplete of macrophages also failed to suppress the generation of cytotoxic activity. The presence of 2-mercaptoethanol during culture may have influenced qualitatively or quantitatively the requirement for macrophages (21).

Cell-cell cooperation in antibody response has been well described (22, 23). More recently, synergy between lymph node and thymus in the graft-vs.-host (GVH) response was established by Cantor and Asofsky (24), who were able to further characterize the collaboration as one involving two subpopulations of T cells. In the same GVH assay system, Tigelaar and Asofsky (25) demonstrated two subpopulations of mouse spleen cells, defined by their lymph node or spleen-seeking properties when injected into irradiated hosts, which also synergized; the activity of both populations was sensitive to treatment with anti-θ serum and complement. At the present time the relationship between these subpopulations of spleen and those defined by nylon column separation and synergy in in vitro sensitization is unclear. No other models have yet been described of synergy between lymphoid subpopulations of a single organ.

The earliest description of lymphoid cell cooperation in an in vitro graft rejection system was that of Lonai and Feldman, a xenogeneic system in which it was speculated that a "nonthymus-processed" cell was interacting with thymus (26). Wagner has described synergy of thymus and an anti-θ-sensitive cell in peripheral lymphoid tissue during the in vitro generation of cytotoxic cells by allogeneic mouse MLC (6). Cohen and Howe (7), Häyry and Andersson (8), and Tigelaar and Feldmann (9) have also reported synergy between thymus and lymph node cells in the in vitro generation of cytotoxic effector cells by MLC. In each case, this is interpreted as a T cell-T cell interaction. A prominent difference between Wagner's results and those discussed here is that Wagner

reports that the synergizing peripheral lymphoid cell (and presumably the thymocyte as well, although not specifically described) is sensitive to the same reagent (anti-θ serum) which eliminates cytotoxic effector activity. In contrast, in the studies reported here, RαMB eliminates effector activity without eliminating the synergy by nylon wool-adherent spleen cells, and preliminary results suggest that allogeneic anti-θ serum has effects similar to RαMB.

In conclusion, the data presented demonstrate synergy between two cell subpopulations of mouse spleen in the in vitro generation of cytotoxic effector cells. Partial characterization has suggested that at least one participating cell is a non-T cell. This cell may be a B lymphycote or a "null" lymphocyte with neither B- nor T-cell characteristics demonstrable. A thymus-dependent cell which is neither sensitive to RαMB nor responsive to PHA, and which is distinct from the cytotoxic effector T cell cannot be excluded. Further studies are being carried out to evaluate these possibilities. Finally, the firm exclusion of a role of macrophages is complicated by the limitations of methods currently available for isolating or depleting selectively for this population. The present evidence, however, supports a role of a synergizing non-T cell which does not appear to be a macrophage.

Summary

Two subpopulations separated from normal spleen have been shown to synergize as responding cells in the in vitro induction of specific cell-mediated cytotoxicity during the mixed lymphocyte culture (MLC). The synergizing populations are a nylon wool column-adherent and a nylon wool column-nonadherent fraction, enriched for B lymphocytes and T lymphocytes, respectively. When a mixture of these fractions is used as the responding cell population in MLC, greater cytotoxicity is generated than would be expected from the sum of activities generated in the two subpopulations sensitized separately. The synergy appears to occur at the sensitization rather than the effector phase. The synergizing cell which is contained in the nylon-adherent subpopulation is distinct from the cytotoxic effector T lymphocyte, is resistant to lysis by rabbit antimouse brain serum, and is unresponsive to phytohemagglutinin; its synergizing function could not be replaced by either plastic-adherent spleen cells or peritoneal exudate cells. These results suggest a role of a non-T-cell nonmacrophage population in the generation of cytotoxic activity.

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