SYNERGY AMONG RESPONDING LYMPHOID CELLS IN THE ONE-WAY MIXED LYMPHOCYTE REACTION

Interaction Between Two Types of Thymus-Dependent Cells*

By WOLFGANG TITTOR,‡ MARIA GERBASE-DELIMA, and ROY L. WALFORD

(From the Department of Pathology, UCLA School of Medicine, Los Angeles, California 90024)

(Received for publication 18 December 1973)

Cantor and Asofsky (1, 2) demonstrated that the inoculation of mixtures of peripheral lymph node (LN)[1] and thymus cells from parental mice into F_1 hybrid recipients would produce graft-vs.-host (GVH) reactions (3) greater than the sum expected from separate reactivities of the two cell populations alone. Studies with cells from thymectomized donors (2) or cells pretreated with antitheta serum (4) indicated that both cell populations involved in this "synergy" were thymus derived. The distribution of the populations among different lymphoid "compartments" and their in vivo susceptibilities to heterologous antithymocyte serum (ATS) suggested that the two T-cell types had different migratory patterns (2). Those present in excess in thymus and spleen (T_1 cells) appeared to recirculate slowly and to be precursors of the cells which inflict immunologic injury in the GVH reaction (2). This ability to inflict injury could be amplified by interaction with rapidly recirculating (T_2) lymphocytes from peripheral blood and lymph nodes. Additional evidence that the synergizing subpopulations resided chiefly in different lymphoid organs was based upon GVH reactivity of LN and spleen cells from lethally irradiated mice reconstituted with syngeneic spleen cells (5).

The mixed lymphocyte culture reaction (MLR) may well represent an in vitro correlate of the early phase of the GVH reaction (3). Synergistic in vitro responses between thymus-dependent subpopulations in MLR and also in the cell-mediated lymphocytotoxic reaction have recently been described in several laboratories (6–8). In the present study of the "one-way" MLR we describe various further parameters of the synergy obtained when responding LN and spleen cells are combined with thymus cells or with each other. Our data lend additional support to the concept that the same two cell types demonstrating synergy in the GVH reaction (T_1 and T_2 cells) also synergize in the MLR.

Materials and Methods

Animals.—4- to 8-mo old male C57BL/6J (*H-2*^{b}), 3- to 10-mo old male CBA/J (*H-2*^{k}), 6- to 7-mo old male (C57BL/6J × 129) F_1 hybrid, and 4- to 6-mo old male (C57BL/6J × CBA/J)F_1 hybrid mice were used.

* Supported by National Cancer Institute grant CA12788.

‡ Recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

[1] *Abbreviations used in this paper*[7] ATS, antithymocyte serum; B6, C57BL/6J mice; GVH, graft-vs.-host; LD, lymphocyte defined; LN, lymph node; MLR, mixed lymphocyte culture reaction; NRS, normal rabbit serum; SD, serologically defined.

Cell Suspensions.—Suspensions from thymus, spleen, and lymph nodes (pooled from inguinal, axillary, brachial, and submandibular nodes) were prepared by teasing in a small amount of medium. Hepes-buffered medium RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) with 1% antibiotic-antimycotic mixture (Gibco) and 7% pooled heat-inactivated human serum was employed (9). Debris was removed by sedimentation, and the cells were washed twice and resuspended in medium. Erythrocytes in spleen suspensions were lysed with ammonium-chloride solution (10). All suspensions were passed through no. 27 gauge needles, examined for viability via trypan blue exclusion, and adjusted to the desired concentration of viable cells.

MLR.—Stimulating cells consisted of CBA spleen cells for the allogeneic reaction and syngeneic cells for controls. 15×10^6 viable stimulating cells/ml were incubated in a 50 μg/ml solution of mitomycin-C for 20 min at 37°C in a water bath, followed by three washes in medium. The MLR itself was carried out in titer plates (220/24A Disco "U" plates, Scientific Educational Products Corp., New York). 0.1 ml of stimulating and 0.1 ml of responding cell suspensions were placed in each cup and the plates sealed with clear mylar covers (35PSM, Linbro Chemical Co., New Haven, Conn.). After 48 h at 37°C, 3 μCi [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co. sp act 1.9 mCi/mmol) in 0.1-ml serum-free medium was added to each cup and incubation continued for another 16 h. The reaction was stopped by chilling to 4°C, the mixtures transferred to centrifuge tubes, washed twice in 5% trichloracetic acid, once in methanol, and transferred to counting vials. After evaporation of methanol, 10 ml of scintillation liquid (5 g 2,5-diphenyloxazole and 100 mg 1,4-bis[2-(5-phenyloxazolyl)]-benzene/liter of toluene containing 3% "solubilizer" [Nuclear-Chicago Corp., Des Plaines, Ill.]) was added. The vials were allowed to stand for 12 h and the [^{3}H]thymidine uptake was measured in a Beckman model LS-25 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Control values from syngeneic combinations were subtracted from the experimental (allogeneic) values. All tests were done in quadruplicate and means and standard errors computed. Statistical comparisons were done via Student's t test. When the "expected" was compared with the observed value, the SE of the observed value was assumed also for the expected one: this introduced a slight conservative bias in the probability values.

In Vitro Treatment with Anti-θ Serum and Complements—Anti-θ serum was produced by injections of AKR mice with CBA thymocytes (10). The activity could be completely absorbed with mouse brain. For in vitro treatment, 25×10^6 living LN cells/ml were incubated in 0.4 ml of anti-θ serum or in normal AKR serum for 30 min at 37°C, then for 30 min at 37°C with 0.6 ml of complement added. The serum of New Zealand rabbits previously screened for low natural toxicity for mouse thymus and spleen cells and also preabsorbed at 4°C with lymphoid cells was employed as the complement source (11).

ATS.—ATS was prepared by immunizing young adult New Zealand white rabbits with C57BL/6J (B6) thymocytes according to Levey and Medawar (12). It was inactivated at 56°C for 30 min and absorbed with 1/20th vol of washed B6 erythrocytes.

Irradiation.—Exposure of mice to 1,200 R whole body irradiation was done with a Picker Vanguard machine (Picker Corp., Cleveland, Ohio) under conditions of 280 kV, 20 mA, half-value layer 1.28 mm copper, at a distance of 52 cm and an administered dose rate of 137 R/min.

RESULTS

MLR Standard Curves for Thymus Cells, LN Cells, and a Thymus-LN Cell Mixture.—The reactivities of different concentrations of responding thymus and LN cells from B6 mice in the standard MLR are shown in Fig. 1. Stimulating cells consisted of mitomycin-treated CBA spleen cells at a constant concentration of 8×10^6 viable cells/ml. Hence the ratio of responding to stimulating cells was allowed to increase with increasing concentration of responding

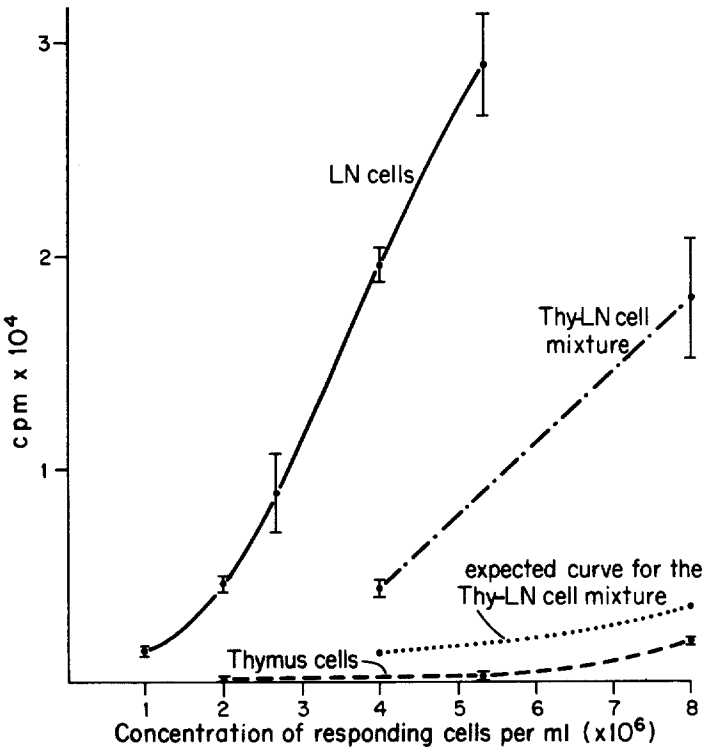


FIG. 1. [^{3}H]thymidine uptake in cpm of thymocytes, LN cells, and a mixture of 80% thymocytes and 20% LN cells from 6-mo old B6 mice plotted against concentration of responding cells. The expected reactivity for the thymus (Thy)-LN mixture was constructed using the reactivity curves for equivalent numbers of thymocytes and LN cells. The sum of cpm values of the participating partner cells was significantly less than the actually observed [^{3}H]thymidine uptake of the mixture (shown at two concentrations). The vertical bars in this and all further figures represent SE.

cells. [^{3}H]thymidine uptake was plotted against the concentration of responding cells. The reactivity curves are consistent with a linear relationship between thymidine uptake and responding cell concentration over much of the curves.

The LN cells were 40–50 times more active than thymus cells. The reactivity of a mixture of thymus cells and LN cells in a 4:1 ratio was measured at two concentrations (Fig. 1) and was four to five times greater than expected, representing "synergy" in the MLR ($P < 0.01$ at the concentration of $8–10^6$ cells/ml). The expected [^{3}H]thymidine uptake was calculated by summing the separate reactivities of thymocytes and LN cells at the corresponding cell concentrations. Pretreatment of either LN or thymus cells with mitomycin-C abolished synergy.

The Effects of Anti-θ Serum upon the Reactivity of LN cells and upon the Syner-

gistic Activity of LN Cells and Thymocytes.—LN-cell suspensions from B6 mice plus complement were incubated in vitro either with AKR anti-θ serum at different dilutions or in 1:4 diluted normal AKR control serum. The suspensions were washed and the LN cells set up in the MLR either alone or with added B6 thymocytes at a ratio of 1:4. Relatively low concentrations of anti-θ (1:128) abolished the capability of LN cells both to respond and to synergize (Table I).

The Effect of Prior Treatment with ATS on the Capacity of LN Cells, Spleen Cells, and Thymocytes to React in MLR—Lymphoid cells were obtained from 8-mo old (B6 × 129)F_1 females which had received intraperitoneally either 0.75 or 0.075 ml of ATS 3 days previously. Their reactivities were compared to

TABLE I

*MLR of LN cells and Thymus (Thy)-LN Cell Mixtures before and after Treatment with Anti-θ Serum**

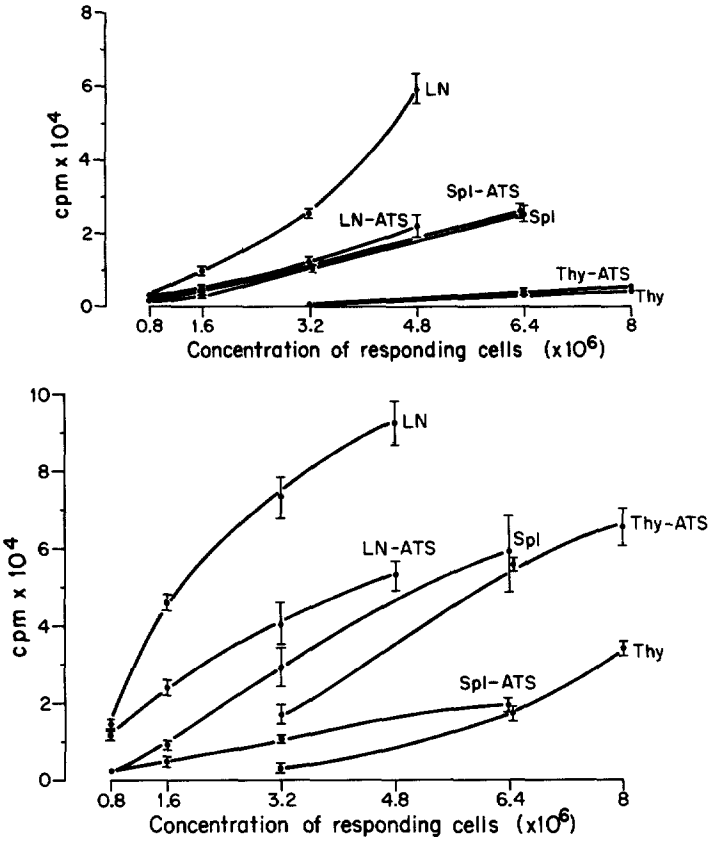
Reactants	Reactivity of untreated cells	Reactivity after treatment with anti-θ serum of the indicated dilutions					
		$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{128}$	$\frac{1}{512}$	$\frac{1}{2,048}$
LN	65,963±6,771	786±276	1,256±276	505±55	4,315±1,104	80,610±13,686	77,351±6,683
Measured value							
% activity compared to untreated cells	—	1.1	1.9	0.7	6.5	122	117
Thy-LN mixture							
Measured value	17,807±5,322	4,305±165	937±482	3,810±1,072	2,875±918	21,311±1,892	17,536±3,285
% activity compared to untreated cells	—	24.1	5.2	21.4	16.1	120	99

*Values are expressed as cpm ± SE. Treatment of the LN cells with anti-θ in a dilution of 1:128 or lower greatly reduced or abolished both their direct reactivity and their ability to synergize with thymocytes. Normal AKR serum at a dilution of 1:4 had a slight inhibitory effect upon the reaction but none at higher dilutions. The Thy-LN mixture was at a 4:1 ratio.

lymphoid cells from control animals treated with normal rabbit serum (NRS). Both doses of ATS decreased the reactivity of LN cells by 30–50% (Figs. 2 and 3), whereas the reactivity of spleen cells was decreased only by the higher dose. A paradoxical effect on thymus cells of animals treated with the highest ATS dose was observed. The reactivity increased two to three-fold. This observation of a reciprocal effect on thymocytes and spleen cells parallels Cantor's and Asofsky's findings on the effect of ATS on the GVH reaction of cells derived from these sources (13).

LN cells obtained from mice pretreated with 0.075, 0.25, and 0.75 ml ATS showed much less [^{3}H]thymidine uptake in combination with normal thymocytes than did LN cells from NRS-treated animals, i.e., ATS decreased or abolished the ability of LN cells to synergize with thymocytes (Fig. 4).

Reactivities of LN and Spleen Cells from Irradiated Mice, and from Irradiated Mice Reconstituted with Syngeneic Spleen Cells.—24 (B6 × 129)F_1 male mice



FIGS. 2 and 3. MLR of LN cells, Spl cells (Spl), and thymocytes of NRS- and ATS-treated 8-mo old B6 mice at different concentrations of responding cells. In Fig. 2 the animals were treated with 0.075 ml NRS or ATS 3 days before the test, in Fig. 3 with 0.75 ml. At the higher dose ATS exerted a depressing effect upon MLR of LN cells and spleen cells; however, at the lower dose only the reactivity of LN cells was depressed. Thymocytes demonstrated a paradoxical pattern. Only the higher ATS dose influenced their reactivity which was increased two- to threefold.

5-mo old were irradiated with 1,200 R. 2 h later 10 of these were injected via the tail vein with 100×10^6 spleen cells from 5-mo old syngeneic males. The MLR of cells from spleen and LN of both irradiated and irradiated spleen cell-reconstituted groups were measured 20 h after irradiation, and compared to cells from untreated animals. Irradiation essentially abolished the reactivities of both LN and spleen cells. The irradiated reconstituted group regained some reactivity, about 17% for LN and for spleen cells about 10% of that of cells from normal animals. Reconstitution thus resulted in populations in both LN and spleen with impaired ability to react in MLR.

LN cells from irradiated but not reconstituted animals possessed little or no capacity to synergize with normal thymocytes (Fig. 5). The LN cells from ir-

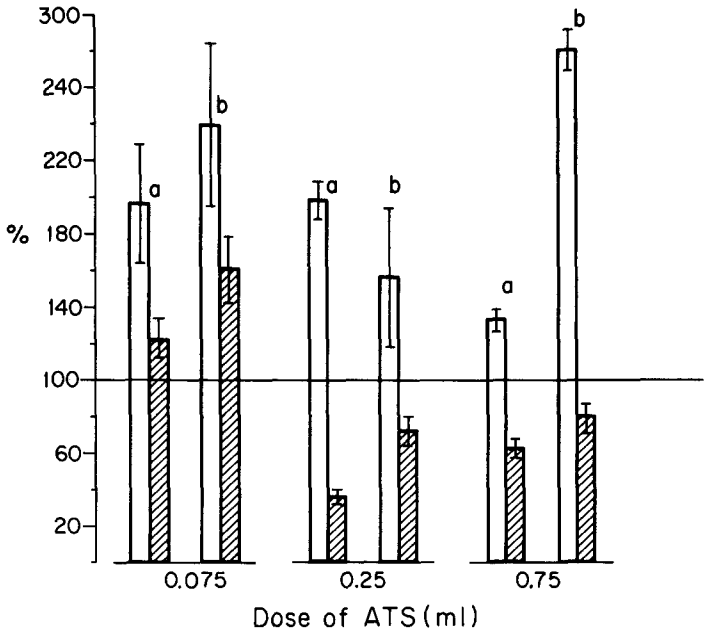


FIG. 4. MLR of Thy-LN mixtures in percent of expected values. Thymocytes were derived from animals treated with NRS, LN cells from NRS-treated (blank column) or ATS-treated (hatched column) mice. Thy/LN ratio was 4:1 in all instances. ATS treatment at the three tested doses abolished the capability of LN cells to synergize with thymocytes. a, 8×10^6 and b, 4×10^6 responding cells/ml.

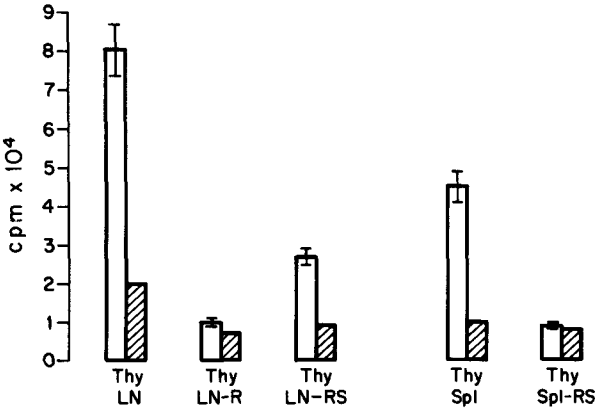


FIG. 5. MLR of 4:1 mixtures of normal thymocytes with normal LN cells or normal Spl cells, or with LN cells derived from irradiated animals (LN-R), or with LN or Spl cells from irradiated and Spl cell-reconstituted mice (LN-RS and Spl-RS). The hatched columns represent the expected values. In irradiated animals the reconstituting Spl cells migrated partially to lymph nodes and partially to spleens. However, only those which migrated to the LN were able to react synergistically with thymocytes. All animals in this experiment were (B6 × 129)F_1 hybrids.

radiated spleen cell-reconstituted mice did synergize with thymocytes. By contrast, while normal spleen cells participated well in synergy, the spleen cells from irradiated spleen cell-reconstituted mice clearly did not synergize when mixed with normal thymocytes. Hence, irradiation and reconstitution with spleen cells resulted in two lymphoid populations (in LN and spleen), neither of which reacted well by itself in the MLR, and only one of which (from LN) showed good synergistic activity in combination with thymocytes (Fig. 5).

Reactivities of Mixtures of Normal Spleen and LN Cells, and of Spleen and LN Cells from Irradiated Animals with and without Reconstitution by Spleen Cells.—The response of a normal spleen-LN-cell mixture was significantly elevated compared to expected values, demonstrating synergy among these cell populations (Fig. 6). Mixtures of spleen and LN cells from irradiated

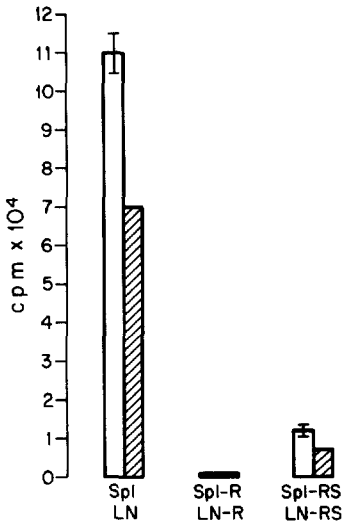


FIG. 6. MLR of 4:1 mixtures of normal Spl cells with normal LN cells, of Spl-R and LN-R cells from mice, and of Spl-RS and LN-RS cells from mice. The hatched columns represent the expected values.

animals were nonreactive; however, mixtures from irradiated spleen cell-reconstituted animals showed some evidence of synergy, although the total response was only 16 to 28% of that of normal spleen cells. These results were obtained with cells from (B6 × 129)F_1 hybrid mice. A mixture of normal spleen and LN cells from B6 mice did not manifest synergy, even though (Fig. 1) thymus and LN cells of this strain do synergize.

DISCUSSION

Our results confirm and extend prior observations that two thymus-dependent cell populations interact synergistically in the MLR (6, 8). The interaction

can be demonstrated at various ratios of total responding to constant stimulator cell numbers (Fig. 1), also when the total number of responding cells is held constant but the ratio of the T-cell subpopulations allowed to vary (8), or when the concentrations per ml of both responding and stimulating cells are allowed to increase (8). Synergy in the MLR is clearly analogous to that described by Cantor and Asofsky and coworkers (1, 2, 5) using the GVH reaction as an indicator system. They described so-called precursor T_1 cells in the thymus and spleen of Balb/C mice. The GVH reactivity attributed to these T_1 cells could be increased by interaction with so-called amplifier or T_2 cells from peripheral blood or lymph nodes. It is pertinent to state in what ways the synergy we observed in MLR is similar to that described for the GVH reaction, and whether any apparent differences exist.

Similarities may first be emphasized. Our experiment using combinations of thymocytes and LN cells in which either one of the populations consisted of F_1 hybrid cells derived from mice of the B6-responding strain and the CBA-stimulating strain indicated that in the MLR model (8), just as in the GVH model (1), both responding populations must be allogeneic to the stimulating cells to participate in synergy. Also, synergy was not observed if either responding cell population was inactivated, via freezing and thawing by Cantor et al. (14), or in our experiments by pretreatment with mitomycin-C.

Cantor (4) demonstrated that both amplifier (T_2) and precursor (T_1) cells involved in synergy in the GVH reaction were θ (+) cells, and that normal LN cells contained both types of cells, although $T_2 \gg T_1$. The amplifier activity of the LN cells was slightly less susceptible to low doses of anti-θ serum than was the overall GVH reactivity. In our MLR experiments we noted that treatment of LN cells with increasing concentrations of anti-θ resulted in loss of both MLR responding and synergizing activities concurrently (Table I). Since different mouse strains and test situations were being employed, we do not regard this lack of a differential effect of anti-θ serum in MLR as necessarily a significant difference.

In the GVH model the spleen appeared to contain both T_1 and T_2 cells ($T_1 \gg T_2$), and these showed different migratory patterns when injected into lethally irradiated mice (5). T_1 cells preferably went to the spleens of the irradiated mice and T_2 cells into the recirculating lymphocyte pool, which included the lymph nodes. Thus, the spleen cells from lethally irradiated spleen cell-reconstituted mice should represent a pure T_1-cell population and LN cells a pure T_2-cell population. Therefore, the LN cells but not the spleen cells of reconstituted mice should synergize with normal thymocytes. This was exactly what we obtained in our MLR model, as shown in Fig. 5. Spleen cells of normal mice of the appropriate strain synergize with thymocytes. Thus, the spleen contains two subpopulations active in both GVH reaction and MLR and these demonstrate similar migratory patterns upon injection into irradiated recipients. The parallel here between GVH and MLR results was not however absolute. In the GVH reaction the combination of spleen and LN cells in a ratio of

5:1 from irradiated reconstituted mice gave reactivity comparable to normal spleen cells. In MLR, while reactivity could be demonstrated under these conditions, it was substantially less than that of normal spleen cells.

The major lesion produced by moderate doses of ATS involves the recirculating lymphocytes (15, 16). Itinerant-recirculating spleen lymphocytes (T_2 cell) and residual sessile cells (T_1 cell), which latter are relatively resistant to ATS, participate in the GVH response (2). We found almost no loss of spleen cell reactivity in MLR after treatment with a low dose of ATS, while LN-cell reactivities were markedly decreased (Fig. 2). By contrast, a higher dose of ATS affected both tissues (Fig. 3). Thus, in the MLR two cell types can react, one derived from lymph nodes, which recirculates and is relatively sensitive to ATS, and a second or sessile type from spleens. A paradoxical effect of ATS on thymocytes was observed only when the higher doses of ATS (0.75 ml) was used.

We could not demonstrate a differential effect of ATS or of anti-θ serum upon the mixed lymphocyte reactivity and synergizing capability of LN cells, although this had been shown in the GVH reaction by Cantor and Asofsky (2, 4).

Other possible differences between GVH and MLR models can be mentioned. While in the GVH reaction a clear relationship exists between the response as measured in the Simonsen assay and the logarithms of the cell doses in the range 0.5–20 $\times$ 10^6 cells/mouse (14), we observed in the range of 1–8 $\times$ 10^6 cells/ml in MLR a more or less linear relationship between [^{3}H]thymidine uptake and numbers of responding cells. Above a certain cell concentration (about 12 $\times$ 10^6 cells/ml), no further increase could be obtained under the conditions of our test. Differences in reaction times are also evident when the GVH reaction and MLR are compared. A measurable response in MLR is achieved by the 2nd day, while the GVH reaction usually requires a longer time (9 days in the experiments by Cantor and Asofsky).

The GVH reaction is of course a far more complicated event (17) than the MLR. In the GVH reaction the recognition of antigen by certain lymphocytes is followed by an inflammatory response by the host, by the transformation of reacting donor lymphocytes into proliferating blasts, and by damage to host cells. The MLC reaction is considered to be an in vitro model of merely the recognition or first phase of the GVH reaction (18). Comparing the complexity of events involved respectively in the GVH reaction and MLR, it is not surprising that some apparent differences might be found in the behavior of the thymus-dependent subpopulations responsible for both reactions. However, the similarities are sufficient to suggest that identical subpopulations may be involved.

In a recent study using the cell-mediated lymphocytotoxic reaction against target cells, Wagner (7) noted synergy between thymocytes and peripheral T cells, thus extending an earlier report by Cohen and Howe (6). Wagner found, however, that for cell-mediated cytotoxicity the thymus cells manifested mainly a helper (amplifying) activity, and that peripheral T cells provided the major source for the precursor cells of cytotoxic lymphocytes. This is in fact opposite

to the roles assigned for T_1-T_2-cell interaction in the GVH reaction. One notes that in the mouse the MLR is directed against so-called lymphocyte-defined (LD) antigens, or against LD plus serologically defined (SD) antigens, and the cytotoxic reaction only against SD antigens (7). Our own studies do not bear directly upon the question of which T-cell subpopulation is precursor, which amplifies, but do indicate that all the described parameters of synergy itself may be manifested during the recognition phase of the cellular immune reaction.

SUMMARY

The present studies have shown that two subpopulations of thymus-dependent lymphocytes may act synergistically in the mixed lymphocyte reaction (MLR) in the mouse. One subpopulation was well represented in the young adult thymus and the other in lymph nodes. For optimum synergy, both populations must be allogeneic to the stimulator cells. Pretreatment of either population with mitomycin-C abolished synergy. Anti-θ serum abolished both MLR responding and synergizing activities of lymphoid cells. The two thymus-dependent subpopulations were both present in the spleen, and displayed different migratory patterns when injected into irradiated mice: one population went to spleens of the irradiated mice, the other to lymph nodes. The effects of anti-thymocyte serum on the MLR and upon synergy were assessed. While minor differences exist and are herein described, our overall results strongly suggest that in our experiments with synergy in MLR, we may be dealing with the same T_1- and T_2-cell subpopulations described by Cantor and Asofsky and coworkers (1, 2, 4, 5, 14) as displaying synergy in the graft-vs.-host reaction.

We are indebted to Jess Peagler and Joyce Wilkinson for their technical assistance.

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