REQUIREMENT FOR NON-T CELLS IN THE GENERATION OF CYTOTOXIC T LYMPHOCYTES IN VITRO*

I. Use of Nude Mice as Source of Non-T Cells

By R. M. SCHILLING, R. A. PHILLIPS, and R. G. MILLER

(From the Ontario Cancer Institute and the Department of Medical Biophysics, University of
Toronto, Toronto, Canada)

The mixed lymphocyte reaction (MLR)[1] is widely used as an in vitro analogue model to investigate cellular immunity (1, 2). In such cultures, responder lymphocytes of one mouse strain are plated together with allogeneic or semiallogeneic (2, 3) stimulator lymphocyte populations which have been inactivated by irradiation or mitomycin C. After 3-4 days, antigen recognition can be measured (4, 5) by uptake of [3H]TdR into newly synthesized DNA of the responder cells (mixed lymphocyte culture [MLC] test). Alternatively relative numbers of cytotoxic T lymphocytes (CTL) in the same cultures can be measured on day 4 or 5 using a ^{51}Cr release cytotoxicity test (6, 7). It has previously been shown by many authors (8-11) that macrophages (or A cells) are necessary during the first 24 h of an MLR as measured by the MLC test. Macrophages have also been demonstrated to be required for the generation of CTL (12, 13).

When more sensitive in vitro culture conditions were introduced by including 5×10^{-5} M 2-mercaptoethanol (2-ME) in the standard culture medium (14-19), it appeared that macrophages in vitro have at least two functions: a viability promotion function apparently acting by conditioning of the medium and an antigen presentation function (18). It also became clear that under these improved culture conditions, A-cell depletion methods appeared not to be so efficient as before (17, 19).

In this paper we have taken spleen cells from nude mice as a source of non-T cells, since it is known that such suspensions contain functional A cells (20), but no cytotoxic lymphocyte precursor (CLP) cells (21).

Below a simple assay system is described in which spleen cells from nude mice (nu/nu) are used as a source of non-T cells, and limiting numbers of LN cells from normal littermates (+/+ or nu/+) are used as a source of CLP cells. It is shown that under optimal tissue culture conditions (including fetal calf serum [FCS] and 2-ME), a synergism occurs that is different from a feeder effect. The CTL cells responsible for the cytotoxicity are shown to be derived from the LN component. Specificity experiments involving genetically different responder cell mixtures demonstrated that synergism between the LN cells and the nu/nu spleen cells can occur even across major histocompatibility differences (includ-

[1] Abbreviations used in this paper: BSA, bovine serum albumin; CLP, cytotoxic T lymphocyte precursor; CTL, cytotoxic T lymphocyte; 2-ME, 2-mercaptoethanol; MLC, mixed leukocyte culture; MLR, mixed leukocyte reaction; PBS, phosphate-buffered saline; RAMB serum, rabbit antimouse brain serum.

ing I-region differences). This observation distinguishes our results from similar cell interactions where the interacting cells had to be syngeneic with respect to histocompatibility antigens (11, 22, 23).

Materials and Methods

Mice.   Homozygous athymic nude mice *(nu/nu)* and their heterozygous normal littermates *(nu/+)* were bred in the animal facility at the Ontario Cancer Institute as described earlier (20, 24). Two inbred strains were available: the original *H-2^k* strain RNC-*nu/nu* and an *H-2^b* strain B6-*nu/nu* obtained by backcrossing RNC-*nu/nu* onto the C57BL/6J background (24). The RNC strain was used in generations F_{18}-F_{22} of inbreeding, the B6 congeneic strain in generations F_{11}-F_{14} of backcrossing. F_1 animals, when needed, were obtained by mating RNC-*nu/nu* males with B6-*nu/+* females.

C57BL/6J × DBA/2J (B6D2F$_1$) mice and C3H/HeJ × DBA/2J (C3D2F$_1$) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. DBA/2 StCrlBR mice were purchased from Canadian Breeding Farm, St. Constant, Quebec, and C57BL/6J × C3H/He (B6C3F$_1$) mice from Bio-Breeding Laboratories, Ottawa. Male or female mice were used. A control experiment showed that there was no difference in the cytotoxic immune response with respect to viable cell input between male or female responder cells, although the cell yield per spleen was lower for female mice. Within a given experiment, only male or only female mice were used.

Cell Suspensions and Media.   Spleen cell suspensions or lymph node cell suspensions from 8-12-wk-old mice were prepared aseptically by mincing the organs with scissors and passing them through a wire mesh (60 gauge) into cold "standard medium" (α-MEM (Flow Laboratories, Inc., Rockville, Md.) with 10% (vol/vol) heat-inactivated (56°C for 45 min) FCS, 20 mM HEPES buffer, and 5 × 10^{-5} M 2-ME). The suspensions were always layered over and washed through FCS as described by Shortman et al. (25) to remove aggregates and fine cell debris. The medium for the micro-^{51}Cr release test ("assay medium") was α-MEM containing 10% (vol/vol) heat-inactivated FCS and 20 mM HEPES buffer. No difference in the specific release was found, however, when standard medium was used instead of assay medium throughout the ^{51}Cr release test.

Tumor Cell Lines and ^{51}Cr-Labeling.   H-2^d target cells in the ^{51}Cr release assay were P815 mastocytoma cells (26) which were maintained in ascites form by weekly in vivo passage in DBA/2 mice. To label target cells for the ^{51}Cr release test, ascitic mastocytoma fluid was diluted (approximately 1:5) with PBS and washed once. The pellet was resuspended in PBS, and the cell concentration was estimated using a hemocytometer. For a standard assay (4-5 microtiter plates) 3 × 10^7 mastocytoma cells in 1 ml were labeled with 1 mCi ^{51}Cr as sodium chromate (Radiochemical Centre, Amersham, England) for 50 min at 37°C in 10% CO$_2$ in air. The labeled cells were then washed four times with assay medium at room temperature, and the cell concentration was adjusted to give 5 × 10^5 cells/ml. Of this, 0.1 ml was added to each well in the micro-^{51}Cr release test. Two other target cells, both maintained in tissue culture, were KHT tumor cells *(H-2^k)* which were kindly provided by Dr. D. A. Clark, and EL-4 *(H-2^b)* cells which were obtained from Dr. H. R. MacDonald, London, Ontario. For labeling these target cells, conditions were as described for P815 above.

Viability Test.   All cell viability measurements were done using two fluorescent stains: ethidium bromide, which stains dead nucleated cells; and fluorescein diacetate, which stains viable nucleated cells (27). Using a fluorescence microscope (Zeiss/Oberkochen, West Germany) with incident illumination, the direct percentage of viable nucleated cells could be measured without counting red blood cells. To get good counting statistics, 400 or more cells per sample were always counted.

Irradiation.   Stimulator cells in one-way mixed lymphocyte cultures were heavily irradiated (1,500 rads) using a ^{137}Cs irradiator at a dose rate of 102 rads/min (28). Cell suspensions were kept in standard medium at 4°C during irradiation.

Anti-θ-Serum Treatment.   Two different anti-θ serum preparations were used, a rabbit anti-mouse brain-associated (RAMB) serum as described by Golub (29) and an AKR anti-θ_{C3H} serum as described by Reif and Allen (30). The former was purchased from Cedarlane Laboratories, Hornby, Ontario ("antimouse T-cell serum" lot no. T-111), the latter from Bionetics Laboratories, Kensington, Md. (lot 231-55-1). The unabsorbed RAMB antiserum had a 50% lysis titer of 1:32, the mouse

anti-θ_{C3H} antiserum a 50% lysis titer of 1:64, both as measured by a ^{51}Cr release assay with LN target cells and simultaneous EB-FDA viability test. After liver absorption (31) specificity controls were done using a titer of 1:10 of either anti-θ serum and testing BM, thymus, or LN. It was found that both sera lysed 1% BM, 99% thymus, and 65–70% LN when tested in an EB-FDA viability test. Functionally, these sera (plus complement) destroyed CLP cells, but lost this activity when absorbed with thymus.

Guinea pig serum ("low-tox," Cedarlane Laboratories) was used as a source of complement. This was absorbed with mouse spleen cells syngeneic to the cell suspension to be treated, filtered through a 0.2-μm Millex filter (Millipore Corp., Bedford, Mass.) and stored at $-20°C$ until use. For anti-θ serum treatment cells were incubated with anti-θ serum (final dilution of 1:10) in 5% BSA-phosphate-buffered saline (PBS) at a concentration of 10^7 cells/ml for 30 min at 4°C. The cells were then washed once with 10 ml of 5% BSA-PBS, resuspended in either 5% BSA-PBS (no complement control) or in a 1:6 dilution of guinea pig serum, incubated at 37°C for 45 min in an atmosphere of humidified 10% CO_2 in air, washed once in 2-ME free standard medium and once in standard medium, and resuspended to the desired concentration in standard medium.

Alloantisera. For one experiment an anti-H-2^b alloantiserum was needed. This was made according to the injection schedule of Gregory et al. (32) with the modification that B6C3F$_1$ spleen cells were injected (instead of B6 cells) into C3H/HeJ animals. The resulting ascites containing anti-H-2^b antibodies was absorbed twice with RNC-nu/+ spleen cells. The 50% lysis point using the treatment protocol, as described for the anti-θ serum treatment, was found to be 1:8 with (B6 × RNC)F$_1$ nu/+ LN cells. Controls with RNC-nu/+ LN cells showed that there was no toxic effect against H-2^k cells at a 1:2 final titer, whereas 98% of F$_1$ cells were killed under these conditions.

In Vitro Immunization. Responder cells (usually 3×10^5–2×10^6 viable cells) were mixed with 2×10^6 nucleated irradiated (1,500 rads) stimulator cells in 75-mm plastic tubes (no. 2058 Falcon Plastics, Oxnard, Calif.) containing 3 ml standard medium. In all experiments the strains for responder and stimulator cells were chosen as to avoid "backstimulation" (3, 33). Thus the stimulator cells were always a hybrid strain (e.g., B6D2F$_1$), whereas the responder cells were one of the parents (e.g., B6 when the target cell was H-2^d). Cultures were set up in duplicate and incubated for 4 days upright (loosely capped) at 37°C in a humidified atmosphere of 10% CO_2 in air.

Micro-^{51}Cr Release Test. A slightly modified micro version (34) of the original ^{51}Cr release was used. This micromodification avoids any centrifugation and allows one to assay for the cytotoxic activity of individual cultures. Briefly, it was performed as follows: The supernate from each culture to be assayed was removed using a specially designed pipette such that the pellet was reproducibly (coefficient of variation of 0.026) left in 0.28 ml medium. The pellet was then suspended, 0.1 ml transferred into the first well of a microtiter tray (Linbro IS-FB-96-TC), and another 0.1 ml transferred into the second well containing 0.1 ml assay medium to give the first dilution. After mixing, 0.1 ml of this well was carried over to the next well for the second dilution, etc. A separate dilution series was made for each culture. Preliminary experiments showed that it was not necessary to wash the cultured cells before the measurement of cytotoxicity. Next 0.1 ml (5 × 10^4 cells) of the labeled target cell suspension (see above) was added to each well and the trays incubated for 4 h at 37°C in a humidified atmosphere of 10% CO_2 in air. Then, 0.1 ml of supernate was collected from each well using a 100 μl Eppendorf pipette placed into a special stand to insure that the distance between the pipette and the bottom of the microtiter well was constant, thus avoiding accidental sucking up of target cells. Control cultures with only responder cells or only stimulator cells were added at a fourfold dilution to 5 × 10^4 target cells to estimate the background ^{51}Cr release, which varied from 6–12% of the total ^{51}Cr incorporated. The total releasable ^{51}Cr was estimated by diluting 0.1 ml of the target cells into 1.5 ml of counting fluid (1% acetic acid in distilled water) which usually gave 75–80% release. Counts were performed in an LKB Wallac 80,000 automatic gamma sample counter. Under the labeling conditions described above, 5 × 10^4 cells gave 8,000–11,000 cpm.

Quantitation of the relative frequency of cytotoxic effector cells was done as described elsewhere (7). Briefly, this is as follows: Specific ^{51}Cr release, p, in each microtiter well was calculated as (observed counts − background)/(total releasable − background). It can be shown (7) that the fraction of target cells lysed is given by $f = 1 - e^{-N\alpha}$, where N is the total number of sensitized cells, α is proportional to the frequency of CTL, and t is the assay time. Under appropriate conditions ($t \gg 1.5$ h, sensitized lymphocyte to target cell ratio not too high), $f = p$, so that $N\alpha$
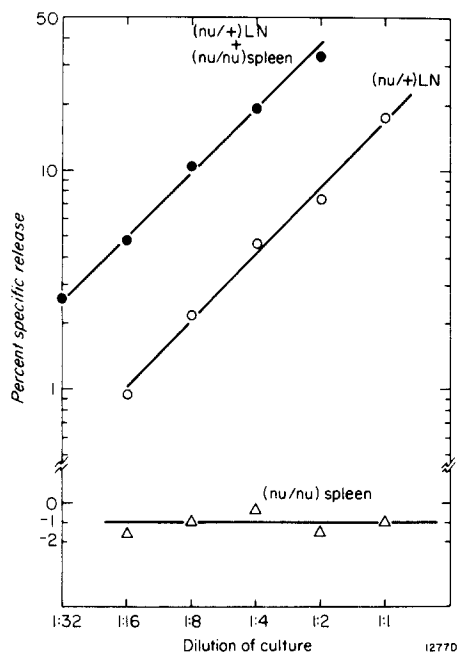
FIG. 1. Titration curves of the ^{51}Cr cytotoxicity test for three different day 4 cultures demonstrating synergism between $nu/+$ LN cells and nu/nu spleen cells. ($\triangle$) 1 $\times$ 10^6 B6 nu/nu spleen cells as responder cells; ($\bigcirc$) 2 $\times$ 10^5 B6 $nu/+$ LN cells as responder cells; ($\bullet$) 2 $\times$ 10^5 B6 $nu/+$ LN cells cocultured with 1 $\times$ 10^6 B6 nu/nu spleen cells. Each culture also contained 2 $\times$ 10^6 irradiated (1,500 rads) B6D2F$_1$ spleen cells as stimulator cells. The target cells were P815 mastocytoma cells.

$= -\dfrac{1}{t} ln (1 - p)$. All of the data below (except for Fig. 1) are presented in terms of Nα rather than p. By comparing Fig. 1 with Fig. 2 or 3 one can see that each titration curve of the type shown in Fig. 1 is represented by a single Nα value, directly proportional to the number of CL in that culture.

Statistical Analysis.   Using the above analysis, each titration curve in the micro-^{51}Cr release test could be reduced to give one Nα value using the best fit to the data points. We observed empirically that titration curves of replicate cultures with small Nα values showed a greater dispersion than replicate cultures with high Nα values. To quantitate this observation we analyzed 53 titration curves, each of which had been done in triplicate or quadruplicate. For each set of replicates, the arithmetic mean Nα and the dispersion (coefficient of variation [CV]) was calculated. A negative linear relationship between the logarithms of the Nα values and the coefficient of variation was found: $\log_{10}$ (CV) = -0.361 $\log_{10}$ (Nα) $-$ 0.925. The correlation coefficient for this regression line was 0.95. We could therefore use this relationship to estimate the CV for the mean Nα of replicate cultures. Error bars shown throughout are 95% confidence limits calculated from this formula. In the remainder of this paper, only Nα values will be presented. Each has been derived from titration curves similar to those shown in Fig. 1. For convenience, they are always given multiplied by 10^4. A numerical value for "cytotoxic activity/culture" refers to this parameter.

Results

Synergism Between Small Numbers of nu/+ LN Cells and nu/nu Spleen. Fig. 1 shows ^{51}Cr cytotoxicity titration curves for three different

cultures. In each culture either 2×10^5 B6-$nu/+$ LN cells, or 10^6 B6-nu/nu spleen cells, or both together were cultured along with 2×10^6 irradiated (1,500 rads) B6D2F$_1$ spleen cells. On day 4, various dilutions of each culture, as shown in the figure, were titrated for their ability to lyse cells bearing D2 alloantigens using ^{51}Cr-labeled P815 cells. It is clear from the figure that the nu/nu spleen cells, although inactive when cultured separately, appear to synergize with $nu/+$ LN cells to give a much larger cytotoxic response than that of $nu/+$ LN cells cultured alone.

Several possible procedures exist for quantitating the activity of each culture and thus assessing more directly the degree of synergism. We have chosen to do this by calculating from each titration curve the relative number of cytotoxic lymphocytes present in the culture according to the procedure outlined in Materials and Methods and described in detail elsewhere (7). This analysis yields a numerical value for a parameter Nα which is proportional to the total number of cytotoxic lymphocytes in the culture. For the $nu/+$ LN curve, Nα = 0.012 and for the $nu/+$ LN + nu/nu spleen curve, Nα = 0.056, or 4.7 times as active.

Fig. 2 shows the results of an experiment in which varying numbers of $nu/+$ LN cells were cultured either alone or mixed with a constant number (10^6) of nu/nu spleen cells. All cultures also contained a constant number of irradiated B6D2F$_1$ spleen cells as stimulator cells. After 4 days of incubation, each culture was titrated separately to give N$\alpha \times 10^4$/culture values. The results show that, for high numbers of $nu/+$ LN cells, addition of syngeneic nu/nu spleen cells has little or no effect on the yield of cytotoxic effector cells. In contrast, for lower numbers of $nu/+$ LN cells per culture, there is a marked synergistic effect.

Fig. 3 shows the results of a similar experiment but in which the nu/nu spleen cells instead of the $nu/+$ LN cells were titrated. Varying numbers of nu/nu spleen cells were cultured either alone or mixed with a constant small (2×10^5) number of $nu/+$ LN cells. The nu/nu spleen cells are themselves inactive at all concentrations but, when cultured with the $nu/+$ LN cells, produce increasing activity with increasing number until a plateau is reached corresponding in this experiment to a synergism of about 10-fold.

Genetic Analysis of Synergism.   The synergistic interaction demonstrated in Figs. 1–3 could be interpreted in two ways: (a) As the number of $nu/+$ LN cells cultured is decreased, a subpopulation of cells necessary in the induction of cytotoxic effector cells is diluted out. This subpopulation is present in nu/nu spleen and is responsible for the synergism. (b) Alternatively, the increased cell density brought about by addition of nu/nu spleen cells improves tissue culture conditions so that small numbers of $nu/+$ LN cells can respond better.

To distinguish between these two possibilities, an approach based on genetic differences in the population cultured was taken. If nu/nu spleen cells have a specific role in the induction of cytotoxic effector cells under the conditions described above, then the synergism should be influenced by the genetic relationship between the nu/nu spleen cells and the stimulator cells. Varying numbers of nu/nu spleen cells from B6 (H-2^b), RNC (H-2^k), and the F$_1$ hybrid between them were tested for their capacity to synergize with constant small numbers of B6 $nu/+$ LN cells. All cultures also contained irradiated C3B6F$_1$
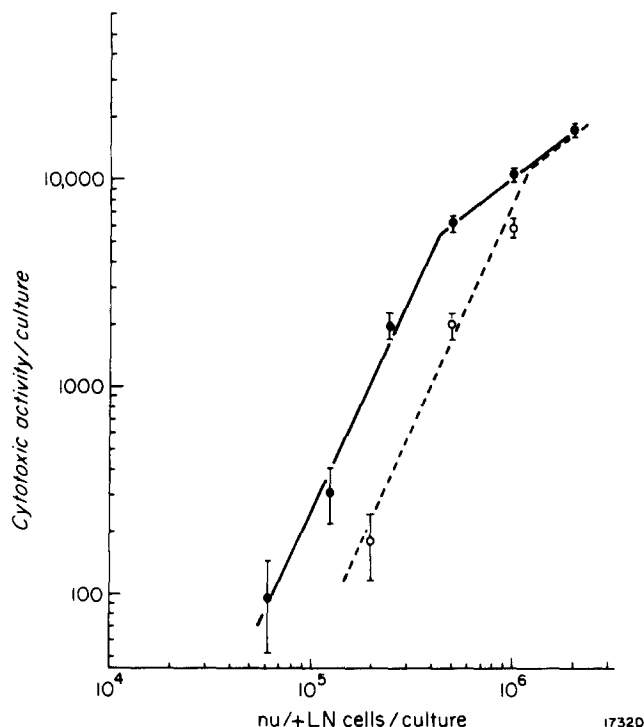
FIG. 2.   Demonstration of synergism in cultures containing as responder cells varying numbers of B6 *nu/*+ LN cells in the presence or absence of 10^6 B6 *nu/nu* spleen cells. (O) *nu/* + LN cells alone; (●) mixtures of varying *nu/*+ LN cells and 1×10^6 *nu/nu* spleen cells. All cultures, done in duplicate, also contained 2×10^6 irradiated (1,500 rads) B6D2F$_1$ spleen cells as stimulator cells. Target cells were P815 *(H-2^d)* mastocytoma cells. Cultures containing 10^6 *nu/nu* spleen cells alone as responder cells gave no activity (not shown).

spleen cells *(H-2$^{b/k}$)* as stimulators and were assayed for cytotoxic activity after 4 days of culture using KHT *(H-2^k)* target cells. If the synergism were due to some feeder effect of the *nu/nu* spleen cells, then all the mixtures should synergize equally well, since all of the cultures contained the same B6 *nu/*+ LN component. The results, shown in Fig. 4, indicate that this is not the case. The mixture of B6 *nu/*+ LN and B6 *nu/nu* spleen shows the largest synergism. This is the only combination in which the mixture is syngeneic and in which both partners can recognize the same alloantigen. A similar phenomenon is seen when varying numbers of B6 *nu/*+ LN cells are cultured with 10^6 *nu/nu* spleen cells from different sources, as is seen in the experiment of Fig. 5.

Attention was focussed on those conditions that produce maximum synergism, i.e., combination of small numbers of *nu/*+ LN cells with large numbers of *nu/nu* spleen cells. One other responding cell mixture was included: RNC *nu/*+ LN and RNC *nu/nu* spleen. Three additional types of stimulator cells and two additional target cells were included. The results of one experiment are shown in Fig. 6.

Several points are worth noting: *(a)* Groups 1-3 repeat the observation of Figs. 4 and 5, i.e. the synergism is obtained only in group 1, in which *nu/nu* and *nu/*+
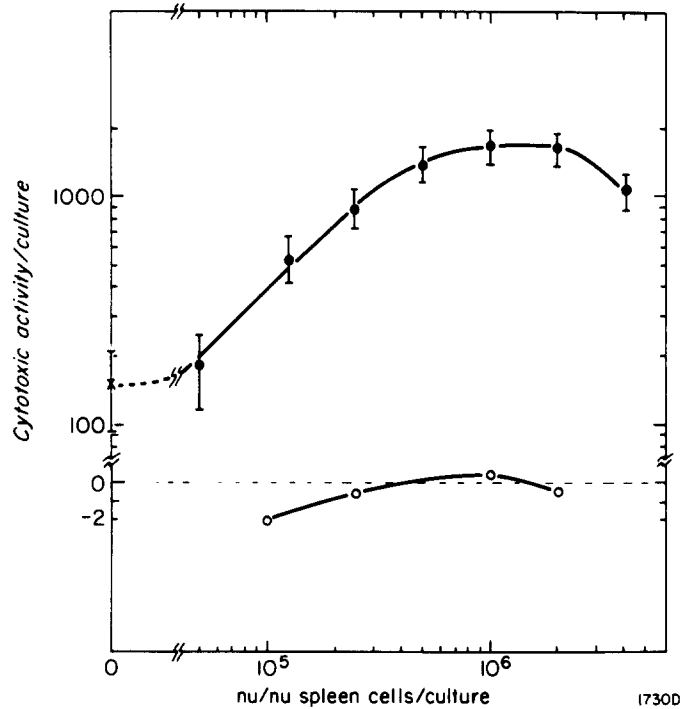
Fig. 3. Demonstration of synergism in cultures containing a constant small number, 2 × 10⁵, of B6 nu/+ LN cells and increasing numbers of B6 nu/nu spleen cells. (X) On ordinate shows activity of 2 × 10⁵ nu/+ LN cells alone; (O) nu/nu spleen cells alone; (●) mixtures of increasing nu/nu spleen cells and 2 × 10⁵ nu/+ LN cells. All cultures also contained 2 × 10⁶ irradiated (1,500 rads) B6D2F₁ spleen cells as stimulator cells and were assayed on day 4 against P815 (H-2ᵈ) mastocytoma target cells.

cells can both recognize the same alloantigen. Note the controls below showing that the synergism in groups 2 and 3 is very small. The cultures of groups 5–7 are identical to those of groups 1–3 except that here we used target cells syngeneic to the other parent of the F_1 stimulator cells, that parent syngeneic to the nu/+ population. All responses are zero, suggesting that addition of nu/nu cells which are syngeneic, semisyngeneic, or allogeneic to the nu/+ cells cannot facilitate recognition of self-antigens. (b) The semiallogeneic and allogeneic responder cell combinations are also active when stimulated by and assayed against third party alloantigens (H-2ᵈ). For example, this is evident when groups 2 and 3 are compared with groups 10 and 11. (c) The collaboration is not a function of the stimulator cells since groups 9–12 (B6D2F₁ spleen as stimulator) are similar to groups 13–16 (P815 as stimulator).

Origin and Nature of the Cytotoxic Effector Cells. The additional cytotoxic effector cells seen in synergizing cultures might be partly derived from nu/nu spleen cells. To test this possibility, cultures of parental and F_1 mixtures of nu/+ LN and nu/nu spleen cells were set up against third party alloantigens. These mixtures have the advantage that one can specifically kill the F_1 cells without harming the other cell partner by using a specific alloantiserum. The results of
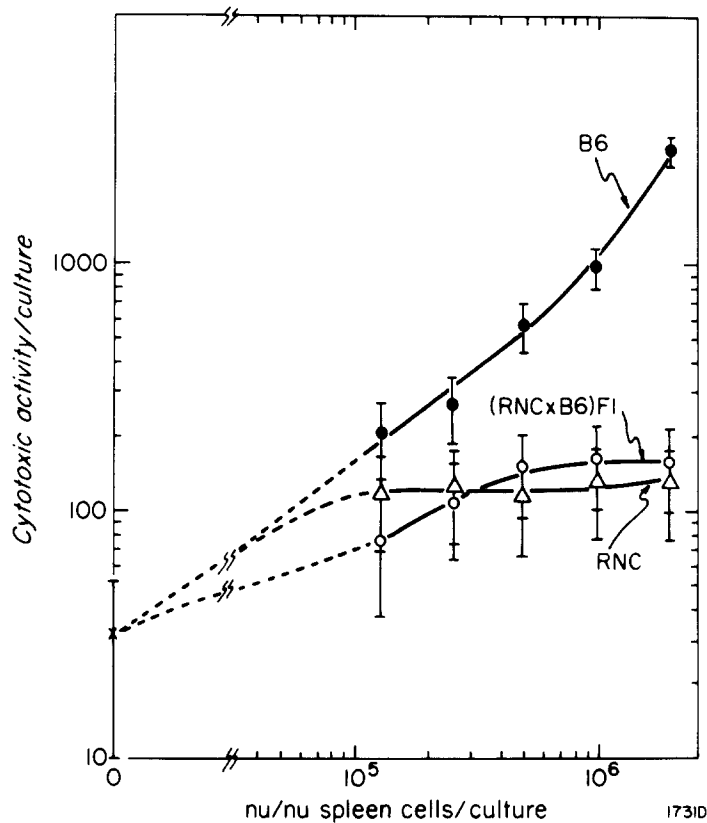
FIG. 4. Demonstration that synergism varies with the genetic origin of the nu/nu spleen cells: nu/nu spleen cell dose-response curve. Constant numbers (2×10^5) of B6 $nu/+$ LN cells and varying numbers of genetically different nu/nu spleen cells were cultured together and assayed on day 4. Stimulator cells for all cultures were irradiated (1,500 rads) C3B6F$_1$ spleen cells. Target cells were KHT $(H\text{-}2^k)$. (X) On ordinate 2×10^5 $nu/+$ LN cells cultured alone. Control cultures with B6, RNC, or F$_1$ nu/nu spleen cells alone (data not shown) gave no activity. (●) Mixture of $nu/+$ LN cells with B6 nu/nu spleen cells; (○) mixture of $nu/+$ LN cells with (RNC $\times$ B6) F$_1$ nu/nu spleen cells; (△) mixture of $nu/+$ LN cells with RNC nu/nu spleen cells.

one experiment are shown in Fig. 7. Here, day 4 cultures were treated with specific anti-H-2^b alloantisera immediately before doing the ^{51}Cr release test. This alloantiserum should destroy cytotoxic activity derived from (RNC $\times$ B6) F$_1$ cells, but not RNC cells. It was observed (Fig. 7) that the cytotoxic activity was destroyed only when the LN cells were of F$_1$ origin. Thus, all the cytotoxic activity is derived from the $nu/+$ LN cells, and no detectable activity comes from the nu/nu spleen cells.

To confirm that in a synergizing culture all the effector cells are T cells, we treated the effector cells with anti-Thy-1.2 (AKR anti-θ_{C3H}) serum before the ^{51}Cr release test. The results (Fig. 8, groups 1-3) show that all the cytotoxic effector cells are Thy-1.2 positive. In the same experiment nu/nu spleen cells were added to $nu/+$ - nu/nu spleen cultures on day 4 just before the ^{51}Cr release test to
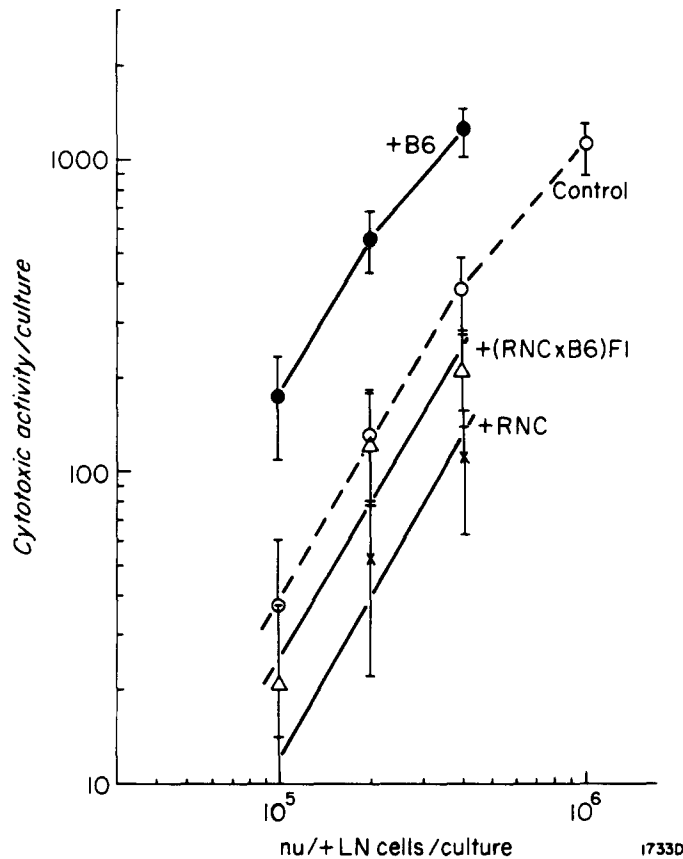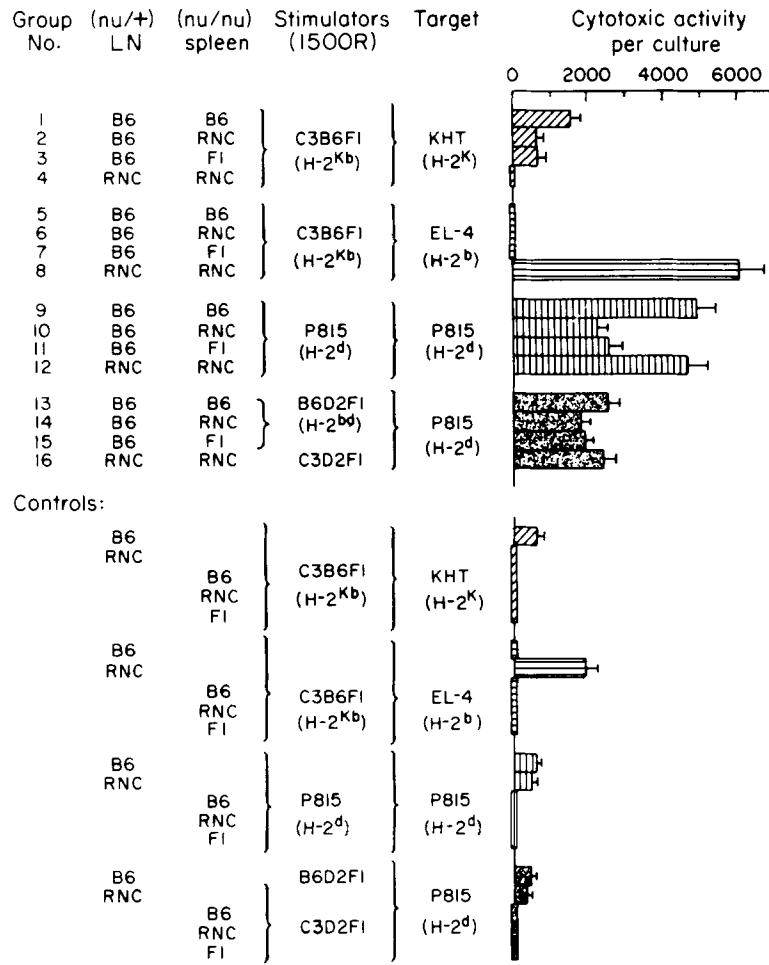
FIG. 5. Demonstration that synergism varies with the genetic origin of the *nu/nu* spleen cells: *nu/+* LN dose response curve. Varying numbers of B6 *nu/+* LN cells and constant numbers (10^6) of genetically different *nu/nu* spleen cells were cultured together and assayed on day 4. Stimulator cells for all cultures were 2 × 10^6 irradiated (1,500 rads) C3B6F$_1$ spleen cells. Target cells were KHT *(H-2^k)*. Control cultures with 1 × 10^6 B6, RNC, or F$_1$ *nu/nu* spleen cells as responder cells showed no activity (data not shown). (O) B6 *nu/+* LN cells alone; (●) mixture of *nu/+* LN cells with B6 *nu/nu* spleen cells; (△) mixture of *nu/+* LN cells with (RNC × B6) F$_1$ *nu/nu* spleen cells; (X) mixture of *nu/+* LN cells with RNC *nu/nu* spleen.

determine whether the *nu/nu* spleen cells must be present for all of the 4-day culture period to obtain synergism. It can be seen (groups 4 and 5, Fig. 8) that late addition of *nu/nu* spleen cells produced no change in the cytotoxic activity over that measured for *nu/+* LN cells alone.

To find out whether the interacting cell types in the induction phase of cytotoxic lymphocyte production from mixtures of *nu/+* LN and *nu/nu* spleen cells were each of T-cell origin, we treated each component separately with RAMB serum before mixing with the other cell component. The cell mixtures were then cultured for 4 days and assayed for cytotoxic activity. As shown in Fig. 9, four different absorption controls were included to test the specificity of the antiserum. Clearly, the cell provided by the *nu/+* LN is a T cell, since the

Group No.	(nu/+) LN	(nu/nu) spleen	Stimulators (1500R)	Target	Cytotoxic activity per culture
I	B6	B6			
2	B6	RNC	C3B6FI	KHT	
3	B6	FI	(H-2^{Kb})	(H-2^K)	
4	RNC	RNC			
5	B6	B6			
6	B6	RNC	C3B6FI	EL-4	
7	B6	FI	(H-2^{Kb})	(H-2^b)	
8	RNC	RNC			
9	B6	B6			
IO	B6	RNC	P815	P815	
II	B6	FI	(H-2^d)	(H-2^d)	
I2	RNC	RNC			
I3	B6	B6	B6D2FI		
I4	B6	RNC	(H-2^{bd})	P815	
I5	B6	FI		(H-2^d)	
I6	RNC	RNC	C3D2FI		

Controls:

	B6 RNC				
		B6 RNC FI	C3B6FI (H-2^{Kb})	KHT (H-2^K)	
	B6 RNC				
		B6 RNC FI	C3B6FI (H-2^{Kb})	EL-4 (H-2^b)	
	B6 RNC				
		B6 RNC FI	P815 (H-2^d)	P815 (H-2^d)	
	B6 RNC		B6D2FI		
		B6 RNC FI	C3D2FI	P815 (H-2^d)	

1735D

Fig. 6. Genetic relationships required to obtain synergism. Cultures contained 2 × 10⁶ C3B6F₁, B6D2F₁, or C3D2F₁ spleen cells or 10⁴ P815 mastocytoma cells as stimulator cells. In the cultures with spleen stimulator cells, 3 × 10⁵ nu/+ LN cells were taken; in cultures with P815 stimulator cells, 6 × 10⁵ nu/+ LN cells. For nu/nu spleen, 8 × 10⁵ viable cells were always taken.

cytotoxic activity of the antiserum is absorbed both by thymus and nu/+ spleen, but not by nu/nu spleen or nu/+ bone marrow. In contrast, when the same batch of absorbed sera was used with nu/nu spleen cells, a different pattern was found (Fig. 9). The unabsorbed serum produced a slight reduction in activity. This does not, however, appear to be due to an anti-Thy-1.2 activity in the serum as nu/nu spleen absorbed this cytotoxic activity as effectively as nu/+ thymus. Similar results were found using a mouse AKR anti-θ$_{C3H}$ serum with the same absorption controls (data not shown). It is concluded, therefore, that the active cell type in nu/+ LN is Thy-1.2 positive, and the active cell in nu/nu spleen is Thy-1.2 negative.

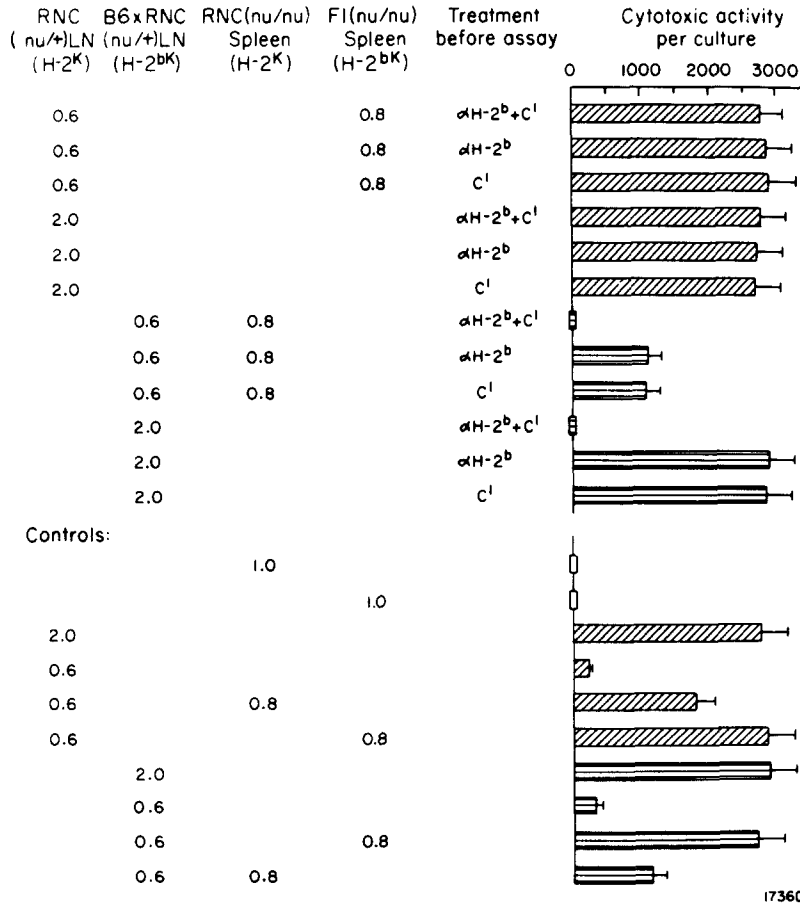| RNC (nu/+)LN (H-2^K)	86xRNC (nu/+)LN (H-2^{bK})	RNC(nu/nu) Spleen (H-2^K)	FI(nu/nu) Spleen (H-2^{bK})	Treatment before assay	Cytotoxic activity per culture
0.6			0.8	αH-2^b+c'	
0.6			0.8	αH-2^b	
0.6			0.8	c'	
2.0				αH-2^b+c'	
2.0				αH-2^b	
2.0				c'	
	0.6	0.8		αH-2^b+c'	
	0.6	0.8		αH-2^b	
	0.6	0.8		c'	
	2.0			αH-2^b+c'	
	2.0			αH-2^b	
	2.0			c'	
Controls:					
		1.0			
			1.0		
2.0					
0.6					
0.6		0.8			
0.6			0.8		
	2.0				
	0.6				
	0.6		0.8		
	0.6	0.8			

17360

Fig. 7. Demonstration using alloantisera that effector cells come from the *nu/+* LN cell population and not the *nu/nu* spleen cell population. Day 4 cultures were treated with alloantiserum and complement to determine the origin of the effector cells. Cell numbers are given in millions of viable cells. Each culture also contained 10^4 irradiated (1,500 rads) P815 mastocytoma cells as stimulators. Target cells in the micro-^{51}Cr release test were P815 cells *(H-2^d)*. The anti-H-2^b serum *(αH-2^b)* was used at a 1:2 dilution, complement at a 1:6 dilution.

Discussion

We have demonstrated that there is a synergism between non-T and T cells in the in vitro generation of CTL under optimized culture conditions which include 2-ME. We interpret our data as indicating that non-T cells play an obligatory role in the generation of CTL from their progenitors (CLP), which are T cells (35). This interaction leads to the activation of CLP and the antigen-specific production of CTL. It is stressed that the interaction between non-T cells and T cells is possible across major histocompatibility differences including *I*-region differences. It is also important to note that the *nu/+* LN may, in fact, provide two unique T cells, one of which is the CLP and the other of which amplifies the response of the CLP (36).

Group No.	nu/+ LN (x10^{-6})	nu/nu spleen (x10^{-6})	Treatment before ^{51}CR release test	Cytotoxic activity per culture (0, 1000, 2000, 3000, 4000)
1	0.2 RNC	1.0 RNC	αθ+C'	(none)
	0.2 RNC	1.0 RNC	αθ	≈3000
	0.2 RNC	1.0 RNC	C'	≈3000
2	0.2 RNC	1.0 FI	-αθ+C'	(none)
	0.2 RNC	1.0 FI	αθ	≈2000
	0.2 RNC	1.0 FI	C'	≈1900
3	2.0 RNC		αθ+C'	(none)
	2.0 RNC		C'	≈4000

Addition of fresh RNC (nu/nu) spleen cells

Group No.	nu/+ LN (x10^{-6})	nu/nu spleen (x10^{-6})	Addition of fresh RNC (nu/nu) spleen cells	Cytotoxic activity per culture
4	0.2 RNC	1.0 FI	0.1	≈2000
	0.2 RNC	1.0 FI	1.0	≈1900
5	0.2 RNC		0.1	≈500
	0.2 RNC		1.0	≈500

Controls:

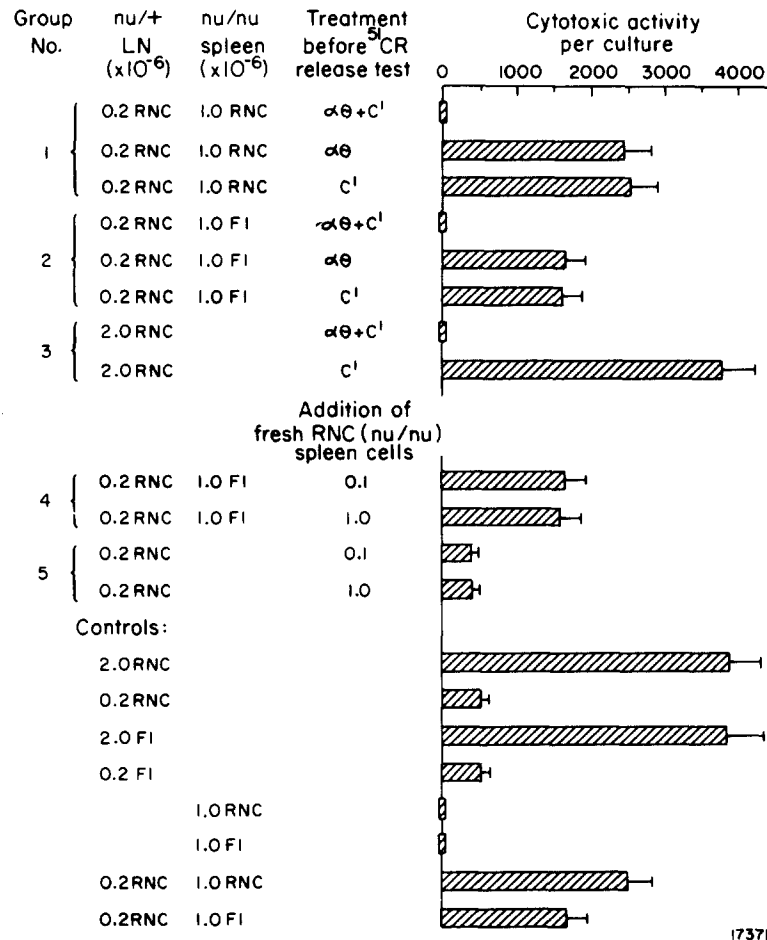| nu/+ LN (x10^{-6})	nu/nu spleen (x10^{-6})		Cytotoxic activity per culture
2.0 RNC			≈3800
0.2 RNC			≈500
2.0 FI			≈3800
0.2 FI			≈500
	1.0 RNC		(none)
	1.0 FI		(none)
0.2 RNC	1.0 RNC		≈2800
0.2 RNC	1.0 FI		≈1600

17370

FIG. 8. Demonstration that all effector cells in synergizing cultures are T cells (groups 1-3), and that addition of *nu/nu* spleen cells to the cultures just before the cytotoxicity assay has no effect on the measured cytotoxicity (groups 4-5). All cultures also contained 2×10^6 irradiated (1,500 rads) C3D2F$_1$ spleen cells as stimulator cells and were assayed using P815 mastocytoma cells as target cells.

Exclusion of Simple Explanations for Synergism. It has been shown by Pierce et al. (18) that macrophages have two functions in an in vitro immune response to Sheep red blood cells (SRBC), a viability promotion function and an antigen presentation function. The viability promotion function can be duplicated by 2-ME as shown by various authors (14-19). Broome and Jeng (16) also showed that 2-ME-like conditioned medium can promote the growth of L1210 cells which otherwise would not grow in Eagle's basal medium. We controlled for these growth-promoting effects by optimizing the FCS concentration and by including 2-ME. Preliminary experiments showed that 10% FCS, 5×10^{-5} M 2-ME, and 3 ml total culture volume were optimal for 4-day cultures as described in this paper. In addition, the genetic studies showed that addition of allogeneic or semiallogeneic nude spleen cells to *(nu/+)* LN cells increased CTL in only

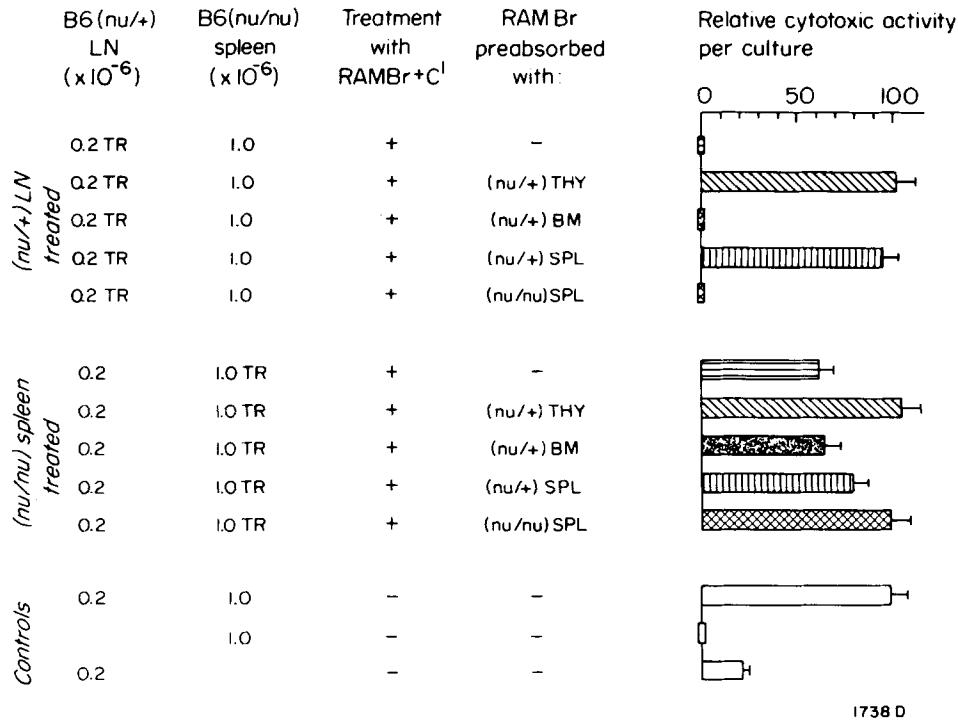| B6 (nu/+) LN ($\times 10^{-6}$)	B6(nu/nu) spleen ($\times 10^{6}$)	Treatment with RAMBr+C'	RAM Br preabsorbed with:	Relative cytotoxic activity per culture

FIG. 9. Demonstration that treatment (TR) of *nu/+* LN, but not *nu/nu* spleen, with RAMB serum plus complement before setting up cultures abolished cytotoxic effector cell activity. Various absorption controls for the RAMB serum are shown. Synergistic activity of untreated mixtures has been arbitrarily set equal to 100 (corresponding to 5,960 Nα $\times$ 10^4/ culture units). In addition to the cell numbers indicated all cultures also contained 2 $\times$ 10^6 irradiated (1,500 rads) B6D2F$_1$ spleen cells as stimulator cells. Effector cell activity was assayed on day 4 using P815 mastocytoma cells.

some cultures in a manner inconsistent with the sole effect being that of viability promotion (Figs. 4–6). Viability tests of these 4-day cultures revealed that all of the groups had a viability of 30–40%, although both their relative cytotoxicity and total cell yield showed big differences in the same experiment (Figs. 4–6). We conclude, therefore, that under the culture conditions described above, cultures are saturated for the viability promotion function of macrophages and any additional activity must be due to some other mechanism.

One other such mechanism could be a mitogenic effect (37). It is conceivable that allogeneically stimulated nude spleen cells or *nu/+* LN cells produce a mitogen that "amplifies" the CLP activation. However, this does not seem to be the case. Compare, for example, groups 1, 2, and 3 of Fig. 6: cytotoxic activity of groups 2 (both *nu/+* and *nu/nu* receiving *H-2*-difference stimulation) is the same as group 3 (only *nu/+* receiving *H-2*-difference stimulation) and much less than group 1.

Another mechanism of activation, namely backstimulation (3, 33), can be excluded as accounting for the synergism, since all *nu/+* LN - *nu/nu* spleen cell mixtures have been expressly chosen to avoid this phenomenon. In some cases,

this required using P815 cells as stimulator cells (38) instead of spleen cells (e.g., groups 9–12, Fig. 6). The allogeneic responder cell mixtures used (e.g., groups 2, 6, and 10, Fig. 6) are unlikely to be affected by backstimulation, because RNC nu/nu spleen cells lack T cells (24) which would be required to recognize the B6 nu/+ LN cells (3, 24). In these cultures, stimulator-responder backstimulation is controlled by taking F_1 stimulator cells. In Fig. 6, the only groups in which backstimulation might be a problem are groups 14 and 15. These only served as controls in the comparison of groups 9–12 and 13–16 to demonstrate that P815 cells are equivalent to spleen cells in their function as stimulator cells.

We conclude that the cause of the synergism is not of a trivial nature (viability promotion, mitogenic effect, backstimulation), but due to obligatory cell interactions between the nu/+ LN and nu/nu spleen cells.

Origin and Properties of Cytotoxic Effector Cells.   Since it is established that nu/nu spleen cells cannot produce cytotoxic effector cells in MLC cultures (12, 24, and Fig. 3) one might expect a priori that the effector cells in cell mixtures between nu/+ LN and nu/nu spleen are derived from the LN component. On the other hand it might be possible that precursor cells in nu/nu spleen (39) are activated by T cells or by T-cell factors (39–41) from the nu/+ LN cells to differentiate into CTL. Another possibility would be the development of a different effector cell mechanism in addition to CTL, e.g., A cells (42), "armed" macrophages (43, 44), or antibody-dependent lysis (45). All of these possibilities are excluded by the results of Fig. 7, where parent-F_1 cell mixtures were treated with alloantiserum and complement immediately before the micro-^{51}Cr release test. If one of the above mechanisms were active, one should have seen some decrease of the activity when the cell mixture with F_1 nu/nu spleen was treated. However, no change was seen (Fig. 7). On the other hand, all of the activity was destroyed when the cell mixture containing F_1 nu/+ LN cells was treated (Fig. 7). This demonstrates that all the effector cells are derived from the LN cells, but does not bear on the question of what the nature of the effector cells is. In previous publications (13, 46) it was shown that CTL from normal MLR cultures are Thy-1 positive and that they lyse target cells specifically. To test whether or not the effector cells of synergistic cell mixtures are T cells, we treated cell mixtures of LN cells after 4 days of culture with anti-Thy-1 and complement immediately before the micro-^{51}Cr release test. As shown in Fig. 8 (groups 1, 2, and 3) all of the activity was destroyed, indicating that all the cytotoxic activity was due to T cells. In Fig. 6 (compare groups 1, 4, 5, and 8) it is shown that this cytotoxic activity is specifically directed only against target cells carrying the same antigens against which the effector cells have been sensitized. We conclude from this that the effector cells produced appear to be identical with respect to two tested properties (Thy-1 antigen, specific lysis) to CTL developed in normal MLC cultures.

Properties of Active Cells in nu/nu Spleen.   As shown in Fig. 9, treatment with nu/nu spleen-absorbed RAMB serum before culture destroyed all the nu/+ LN cell activity but none of the nu/nu spleen cell activity. Similar results were obtained with liver-absorbed AKR anti-θ_{C3H} serum indicating to us that the active subpopulation(s) in nu/nu spleen are not T cells.

Does the active cell type of nu/nu spleen have specific antigen recognizing

function? This question was tested in Fig. 6. As indicated above, syngeneic responder cell mixtures showed that antigen recognition and target cell lysis were antigen specific (groups 1, 4, 5, and 8 in Fig. 6). Two special cases with semiallogeneic and allogeneic responder cell mixtures were of interest to us because we saw no synergistic response. In the allogeneic responder cell mixture (groups 2 and 6), RNC *nu/nu* spleen cells recognize H-2^b antigens of the F$_1$ stimulator cells, but B6 *nu/+* LN cells recognize H-2^k antigens. We consider that this explains why there is no synergism against either KHT *(H-2^k)* or EL-4 *(H-2^b)* target cells. In the other special case with semisyngeneic responder cell mixtures (groups 3 and 7, Fig. 6) there is no antigeneic stimulation of the F$_1$ *nu/nu* spleen cells since they are syngeneic to the stimulator cells, perhaps explaining why there is no synergism in this combination. In contrast, however, the same responder cell mixtures, when stimulated by a third party antigen which both components of the mixture can recognize, showed marked synergism (groups 10 and 11, Fig. 6).

In conclusion, these data are consistent with the hypothesis that the active cell type of *nu/nu* spleen has specific antigen-recognizing function. Collaboration between the *nu/nu* spleen cells and the *nu/+* CLP requires that both recognize the same antigen. Collaboration is possible across an allogeneic difference including *I*-region differences, but only against a third party antigen.

This finding distinguishes our results from those of Rosenthal et al. (11, 22, 23) who found that, in responding to soluble protein antigens, the cooperation between macrophages and T lymphocytes of guinea pigs required identity at the major histocompatibility locus. The difference may be related to different requirements for responses to H-2 antigens and soluble protein antigens.

As shown in Fig. 2, synergism occurs only at limiting numbers of *nu/+* LN cells in the slope 2 region of a log-log plot. The observation that addition of *nu/nu* spleen cells changes slope 2 of a log-log plot into slope 1 (Fig. 2) suggests that the cell type being diluted out in *nu/+* LN is the same as the non-T cell in *nu/nu* spleen accounting for the synergism (47).

Studies of the active cell in *nu/nu* spleen using velocity sedimentation cell separation, coupled with measurements of radiosensitivity and adherence properties indicate[2] that the major activity is due to a relatively radiation sensitive, nonadherent cell population with modal sedimentation velocity of 4.2 mm/h. In addition, there is a minor subpopulation of relatively radioresistant active cells with modal sedimentation velocity of about 6 mm/h. The observed sedimentation profiles are inconsistent with the active cells being B cells (48), but have similarities to the profiles measured for A cells (49, 50). Similar active cells can be seen in the spleen, LN, and peritoneal cells of normal mice (work in progress).

Summary

The ability of small numbers of LN cells to produce cytotoxic lymphocytes on in vitro culture with allogeneic stimulator cells is greatly augmented by the addition of spleen cells from athymic nude mice. The possibility that the synergism is a result of improved culture conditions or a "feeder effect" is

---

[2] Miller, R. G., R. M. Schilling, and R. A. Phillips. Manuscript in preparation.

excluded. All cytotoxic cells found in these cultures are shown to be T cells and to arise from precursors contained in the LN-cell component. The nude spleen cell component appears to be providing a required non-T cell which has been lost from the LN component through dilution. Synergism between the two components can occur whether they are syngeneic or allogeneic provided that both can recognize the same alloantigens in the stimulator population.

References

1. Bach, F. H., M. L. Bach, P. M. Sondel, and G. Sundharadas. 1972. Genetic control of mixed leukocyte culture reactivity. *Transplant. Rev.* 12:30.
2. Häyry, P., L. C. Andersson, S. Nordling, and M. Virolainen. 1972. Allograft response *in vitro. Transplant. Rev.* 12:91.
3. von Boehmer, H. 1974. Separation of T and B lymphocytes and their role in the mixed lymphocyte reaction. *J. Immunol.* 112:70.
4. Dutton, R. W. 1965. Further studies of the stimulation of DNA synthesis in cultures of spleen cell suspensions by homologous cells in inbred strains of mice and rats. *J. Exp. Med.* 122:759.
5. Bach, F. H., and N. K. Voynow. 1966. One-way stimulation in mixed leukocyte culture. *Science (Wash. D. C.).* 153:545.
6. Brunner, K. T., J. Mauel, J-C. Cerottini, and B. Chapuis. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro* inhibition by isoantibody and by drugs. *Immunology.* 14:181.
7. Miller, R. G., and M. Dunkley. 1974. Quantitative analysis of the ^{51}Cr release cytotoxicity assay for cytotoxic lymphocytes. *Cell. Immunol.* 14:284.
8. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. *J. Immunol.* 101:262.
9. Hersh, E. M., and J. E. Harris. 1968. Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leukocytes. *J. Immunol.* 100:1184.
10. Alter, B. J., and F. H. Bach. 1970. Lymphocyte reactivity *in vitro.* I. Cellular reconstitution of purified lymphocyte response. *Cell. Immunol.* 1:207.
11. Rosenthal, A. S., and E. M. Shevach. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* 138:1194.
12. Wagner, H., M. Feldmann, W. Boyle, and J. W. Schrader. 1972. Cell-mediated immune response in vitro. III. The requirement for macrophages in cytotoxic reactions against cell-bound and subcellular alloantigens. *J. Exp. Med.* 136:331.
13. MacDonald, H. R., R. A. Phillips, and R. G. Miller. 1973. Allograft immunity in the mouse. II. Physical studies of the development of cytotoxic effector cells from their immediate progenitors. *J. Immunol.* 111:575.
14. Chen, C., and J. G. Hirsch. 1972. The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells in vitro. *J. Exp. Med.* 136:604.
15. Heber-Katz, E., and R. E. Click. 1972. Immune response *in vitro.* V. Role of mercaptoethanol in the mixed-leukocyte reaction. *Cell. Immunol.* 5:410.
16. Broome, J. D., and M. W. Jeng. 1973. Promotion of replication in lymphoid cells by specific thiols and disulfides in vitro. *J. Exp. Med.* 138:574.

17. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods*. 5:239.

18. Pierce, C. W., J. A. Kapp, D. D. Wood, and B. Benacerraf. 1974. Immune responses *in vitro*. X. Functions of macrophages. *J. Immunol*. 112:1181.

19. Bevan, M. J., R. Epstein, and M. Cohn. 1974. Effect of 2-mercaptoethanol on murine mixed lymphocyte cultures. *J. Exp. Med*. 139:1025.

20. Croy, B. A., and D. Osoba. 1973. Nude mice — a model system for studying the cellular basis of the humoral immune response. *Cell. Immunol*. 9:306.

21. Wagner, H. 1972. The correlation between the proliferative and the cytotoxic responses of mouse lymphocytes to allogeneic cells *in vitro*. *J. Immunol*. 109:630.

22. Rosenthal, A. S., P. E. Lipsky, and E. M. Shevach. 1975. Macrophage-lymphocyte interaction and antigen recognition. *Fed. Proc*. 34:1743.

23. Shevach, E. M., and A. S. Rosenthal. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J. Exp. Med*. 138:1213.

24. Croy, B. A., and D. Osoba. 1974. Use of genetically defined nude mice in the study of the mixed leukocyte reaction. *J. Immunol*. 113:1626.

25. Shortman, K., N. Williams, and P. Adams. 1972. The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions. *J. Immunol. Methods*. 1:273.

26. Dunn, T. B., and M. Potter. 1957. A transplantable mast-cell neoplasm in the mouse. *J. Natl. Cancer Inst*. 18:587.

27. Takasugi, M. 1971. An improved fluorochromatic cytotoxic test. *Transplantation (Baltimore)*. 12:148.

28. Cunningham, J. R., W. R. Bruce, and H. P. Webb. 1965. A convenient ^{137}Cs unit for irradiating cell suspensions and small laboratory animals. *Phys. Med. Biol*. 10:381.

29. Golub, E. S. 1971. Brain-associated θ-antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol*. 2:353.

30. Reif, A. E., and J. M. Allen. 1966. Mouse thymic isoantigens. *Nature (Lond.)*. 209:521.

31. Gorczynski, R. M. 1974. Evidence for *in vivo* protection against murine-sarcoma virus-induced tumours by T lymphocytes from immune animals. *J. Immunol*. 112:533.

32. Gregory, C. J., E. A. McCulloch, and J. E. Till. 1972. Repressed growth of C57BL marrow in hybrid hosts reversed by antisera directed against non-H-2 alloantigens. *Transplantation (Baltimore)*. 13:138.

33. von Boehmer, H., and J. Sprent. 1974. Expression of M locus differences by B cells but not T cells. *Nature (Lond.)*. 249:363.

34. Dunkley, M., R. G. Miller, and K. Shortman. 1974. A modified ^{51}Cr release assay for cytotoxic lymphocytes. *J. Immunol. Methods*. 6:39.

35. Cerottini, J-C., and K. T. Brunner. 1974. Cell mediated cytotoxicity allograft rejection and tumour immunity. *Adv. Immunol*. 18:67.

36 Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly$^+$ cells in the generation of killer activity. *J. Exp. Med*. 141:1390.

37. Andersson, J., O. Sjöberg, and G. Möller. 1972. Mitogens as probes for immunocyte activation and cellular cooperation. *Transplant. Rev*. 11:131.

38. Lundak, R. L., and D. J. Raidt. 1973. Cellular immune response against tumour cells. I. *In vitro* immunization of allogeneic and syngeneic mouse spleen cell suspensions against DBA mastocytoma cells. *Cell. Immunol*. 9:60.

39. Wortis, H. H., S. Nehlsen, and J. J. Owen. 1971. Abnormal development of the thymus in 'nude' mice. *J. Exp. Med.* **134**:681.

40. Wortis, H. H. 1974. Immunological studies of nude mice. *In* Contemporary Topics in Immunobiology. Plenum Publishing Corp., New York. 243.

41. Scheid, M. P., M. K. Hoffman, K. Komuro, U. Hämmerling, J. Abbott, E. A. Boyse, G. H. Cohen, J. A. Hooper, R. S. Schulof, and A. L. Goldstein. 1973. Differentiation of T cells induced by preparations from thymus and by nonthymic agents. *J. Exp. Med.* **138**:1027.

42. Zighelboim, J., B. Bonavida, and J. L. Fahey. 1974. Heterogenous populations of cytotoxic cells in the peritoneal cavity of Balb/c mice immunized with allogeneic EL4 leukemia cells. *Cell. Immunol.* **12**:280.

43. Evans, R., and P. Alexander. 1971. Rendering macrophages specifically cytotoxic by a factor released from immune lymphoid cells. *Transplantation (Baltimore).* **12**:227.

44. Evans, R., and P. Alexander. 1972. Role of macrophages in tumour immunity. I. Cooperation between macrophages and lymphoid cells in syngeneic tumour immunity. *Immunology.* **23**:615.

45. Van Boxal, J. A., J. D. Stobo, W. E. Paul, and J. Green. 1970. Antibody-dependent lymphoid cell-mediated cytotoxicity: no requirement for thymus-derived lymphocytes. *Science (Wash. D. C.).* **175**:194.

46. MacDonald, H. R., R. A. Phillips, and R. G. Miller. 1973. Allograft immunity in the mouse. I. Quantitation and specificity of cytotoxic effector cells after *in vitro* sensitization. *J. Immunol.* **111**:565.

47. Mosier, D. E., and L. W. Coppleson. 1968. A three-cell interaction required for the induction of the primary immune response *in vitro. Proc. Natl. Acad. Sci. U. S. A.* **61**:542.

48. Miller, R. G., R. M. Gorczynski, L. Lafleur, H. R. MacDonald, and R. A. Phillips. 1975. Cell separation analysis of B and T lymphocyte differentiation. *Transplant. Rev.* **25**:59.

49. Gorczynski, R. M., R. G. Miller, and R. A. Phillips. 1971. *In vivo* requirement for a radiation-resistant cell in the immune response to sheep erythrocytes. *J. Exp. Med.* **134**:1201.

50. Abrahams, S., R. A. Phillips, and R. G. Miller. 1973. Inhibition of the immune response by 7S antibody. Mechanism and site of action. *J. Exp. Med.* **137**:870.