Lymphocyte Cytotoxicity *in vitro*: Activation and Release of a Cytotoxic Factor

WE have reported that cell contact *per se* is not sufficient to explain the rapid and dramatic *in vitro* destruction of target *L* cells observed when they are treated with immune lymphocytes or non-immune phytohaemagglutinin (PHA)-stimulated lymphocytes derived from various inbred mice[1]. Instead, cell contact is apparently involved in the induction of additional steps, which result in the release of a soluble toxic factor(s). The presence of the factor(s) is related to the ability of the aggressor cell to promote the destruction of target *L* cells. Once released, the factor has a non-specific toxic effect when tested on mammalian cells of many genotypes[2]. Lymphocyte-induced destruction of target cells seems to be related to the capacity of the aggressor lymphocyte to undergo enlargement and form blast cells. With these observations in mind we decided to treat lymphocytes *in vitro* by various techniques known to induce blast cells, and to test the culture medium for the presence of cytotoxic material.

Suspensions of small lymphocytes from the spleen and thymus of non-immune *C57Bl/6* and *S/W* mice were prepared by suspending the tissues in Eagle's minimal essential medium (MEM) plus 10 per cent foetal calf serum, cutting it into small pieces and dispersing the cells by repeated pipetting. The resulting suspensions were placed in individual tubes and centrifuged for 1 min at 75*g*. Supernatant fluids were transferred to fresh tubes and centrifuged for 1 min at 135*g*. The cells remaining in the final supernatants were sedimented by centrifugation at 400*g* for 3 min. The final cell pellets were resuspended in MEM. Cell suspensions routinely consisted of 95–100 per cent small lymphocytes, 95 per cent of which were viable.

Lymphocytes were established in separate tube cultures containing 10×10^6 cells in 2·0 ml. of MEM and one of the following materials: (1) 30 μg of PHA-P (Difco); (2) 5·0 μg of poly-L-lysine (K and K Chemicals, California), or (3) 0·1 ml. of rabbit anti-mouse serum. This antiserum was produced in rabbits by the following method: serum was collected from three rabbits, 5 days after the last of five weekly injections of *A/Jax* and *C3H* mouse tissue, pooled and heat-inactivated by treatment at 56° C for 1 h. The serum pool had a haemagglutinin titre of 2^9, determined by the saline double dilution method against mouse red blood cells. Treated and untreated control lymphocyte cultures were incubated for 48 h at 37° C.

After incubation, cells and cell debris were removed from the cultures by centrifugation at 1,000*g* for 20 min and passage through a 0·45μ 'Millipore' filter or both. The cell pellets were examined under the light microscope for the presence of enlarged lymphocytes and discarded. The resulting supernatant fluid was placed on fresh individual *L* cell cultures (250,000 cells/tube) and incubated at 37° C. These cultures were removed after 48 h, examined microscopically and their capacity to incorporate ^{14}C-amino-acids into cell protein was measured by the following method. Pre-warmed labelling MEM (2 ml.) containing 0·30 μCi/ml. of ^{14}C protein hydrolysate was added to each tube. After 5 min at 37° C the tubes were chilled to 0° C in an ice bath, centrifuged at 400*g* for 1 min and the MEM was discarded. Cell protein was extracted by disrupting the cells in 0·1 M KOH and precipitating the protein at 95° C with one and a half volumes of 10 per cent trichloroacetic acid. The protein was made soluble with alkali, precipitated in TCA, and pelleted by centrifugation three times. The final protein precipitates were collected on individual 'Millipore' filters, air dried, placed in 10 ml. of toluene scintillation fluid containing 4 g of PPO/l. and 50 mg of POPOP/l. and counted for 10 min in a scintillation counter.

Table 1. RELEASE OF CYTOTOXIC FACTOR (LCF) BY "ACTIVATED" LYMPHOCYTES IN THE ABSENCE OF TARGET CELLS

Cells	Activating agent	C.p.m. of ^{14}C-amino-acids incorporated into *L* cell protein
Thymocytes *C57Bl/6*	Phytohaemagglutinin (PHA)	8,200
Thymocytes *C57Bl/6*	Xenogeneic antibody	7,705
Lymphocytes *C57Bl/6*	PHA	577
Lymphocytes *C57Bl/6*	Xenogeneic antibody	2,679
Lymphocytes *C57Bl/6*	Poly-L-lysine	7,550
Lymphocytes *C57Bl/6* (heat-killed)	PHA control	7,997
Lymphocytes *C57Bl/6*	Non-treated control	7,876
L cells	PHA control	8,159
L cells	Xenogeneic antibody control	7,973

Cell free medium was obtained from 48 h cultures of non-immune lymphocytes or thymocytes treated with various activating agents and 2·0 ml. placed on fresh *L* cell tube cultures (250,000 cells/tube). After 48 h of incubation, the *L* cells were labelled with 2·0 ml. of ^{14}C-amino-acid hydrolysate, 0·30 μCi/ml., for 5 min, the protein was extracted and the amount of radioactivity was determined in a Beckman *LS*-100 liquid scintillation counter as described in the text.

The results obtained with the lymphocytes derived from *C57Bl/6* mice are shown in Table 1 and are representative of many experiments. PHA and xenogeneic antibody evidently induced *C57Bl/6* lymphocytes to release a substance which is toxic for *L* cells. This material was not present in the medium derived from unstimulated lymphocytes, non-viable lymphocytes treated with PHA or lymphocytes treated with poly-L-lysine. The latter has been reported to promote *in vitro* aggressor–target cell aggregation but not cell destruction[3]. In addition, cytotoxic material was not present in the culture medium derived from thymocytes or *L* cells treated with PHA or Ab. We have demonstrated that non-immune mouse thymocytes are unable to destroy target *L* cells in the presence of PHA or xenogeneic Ab[2]. Microscopy of these tubes revealed that blast cells were only present in the cultures the medium of which was toxic for *L* cells. The amount of ^{14}C-amino-acids incorporated into cell protein in this experiment is a direct reflexion of the number of viable cells in the cultures[1].

It is well known that enlarged blast cells appear when genetically dissimilar non-immune lymphocytes are mixed and cultured together *in vitro*[4,5]. This reaction has been related to the degree of antigenic disparity between the cell donors in studies of both man[6] and experimental animals[7]. This seemed to be an ideal system to ascertain the relationship between the appearance of enlarged blast cells and medium cytotoxicity in the presumed absence of cell associated antibody and non-specific exogenous inducing agents.

Small lymphocytes were obtained from the spleens of non-immune *A/Jax* and *C57Bl/6* mice as described earlier, and established in syngeneic or mixed allogeneic cultures at varying cell ratios. Each tube culture contained a total of 10×10^6 lymphocytes in 2·0 ml. of MEM. After 48 h of incubation at 37° C the cells and cell debris were removed from the cultures as previously described. The supernatant fluids were then placed on fresh *L* cell tube cultures (250,000 cells/tube) and the tubes were incubated at 37° C. After 48 h, each culture was subjected to microscopic examination and the capacity of the cells to incorporate ^{14}C-amino-acids into cell protein was measured, as previously described. Table 2 shows that a toxic substance was present in the medium obtained from mixed cultures but not in medium obtained from unmixed or

Table 2. RELEASE OF CYTOTOXIC FACTOR (LCF) BY "ACTIVATED" LYMPHOCYTES IN MIXED CULTURE

Ratio *C57Bl/6* to *A/Jax* lymphocytes	C.p.m. of ^{14}C-amino-acids incorporated into *L* cell protein
1 : 0	9,477
3 : 1	443
1 : 1	2,601
1 : 3	4,030
0 : 1	8,487
0 : 0 Non-treated control medium	9,038

Cell free medium was obtained from 48 h mixed or unmixed cultures of non-immune *C57Bl/6* and *A/Jax* mouse lymphocytes and 2·0 ml. placed on fresh *L* cell tube cultures (250,000 cells/tube). After 48 h of incubation the *L* cells were labelled for 15 min as described in Table 1.

syngeneic cultures. Similar results have been obtained employing lymphocyte combinations obtained from other mouse strains.

L cells exposed to medium derived from PHA or xenogeneic Ab stimulated lymphocytes or mixed lymphocyte cultures exhibit the same histopathological changes. The cells develop bipolar, perinuclear vacuoles, round up and undergo cytolysis.

This *in vitro* study provides two lines of evidence to support the hypothesis that "activated" lymphocytes release a cytotoxic material(s) and the release is related to the appearance of blast cells.

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Immunogenicity of *L* 1210 Murine Leukaemia Cells after Treatment with Neuraminidase

The privileged position of trophoblast and tumour cells may depend on a barrier which interferes with antigenic expression. As a working hypothesis it was suggested that cell surface sialomucins could act as a barrier to intimate contact between such privileged cells and lymphoid cells. Subsequent studies have indicated that histocompatibility antigens on mouse trophoblast can be detected after treatment with neuraminidase[2]. The increased immunogenicity of Landschutz ascites tumour cells after treatment by the same enzyme has also been reported from this laboratory[3]. This communication reports studies with another murine tumour. *L* 1210 (v) leukaemic cells were passaged every 5 days by 0·05 ml. of ascitic fluid in *C57Bl/DBA2F*$_1$ mice. All passage animals which were not killed died within 8 days of injection, with haemorrhagic ascites and wasting.

For each experiment, contamination of the ascitic fluid by red cells was reduced by exposure to 0·1 N saline for 10–20 s followed by rapid restoration to isotonicity with 10 N saline. The cells were then washed four times in tissue culture medium 199 (Glaxo). Some of the washed cells were incubated in neuraminidase (500 u/ml., 2×10^6 cells/ml.) for 30 min at 37° C and *p*H 5·5 in 0·05 M sodium acetate buffer with calcium chloride. The undiluted enzyme solution had a quoted strength of 500 u/ml. (Behringwerke, batch No. 966B, source *Vibrio cholerae* culture; one unit is the equivalent of the number of μg of *N*-acetylneuraminic acid split off from an acid glycoprotein in this buffer in 15 min at 37° C). Control cells were similarly incubated in the acetate buffer alone and subsequently referred to as untreated cells. After incubation the cells were washed three times in 199 solution. Cell viability was assessed by exclusion of lissamine green and was always better than 90 per cent in the cell suspensions used; loss of viability was not observed after incubation in neuraminidase.

Table 1. Inoculation of *L* 1210 cells into *C57Bl/DBA2* mice

Group	*In vitro* treatment of *L* 1210 cells	Treatment of recipient mice	Outcome
		Inoculation experiments	
1	Acetate buffer	Nil	9/9 dead by day 20
2	Neuraminidase	Nil	9/9 alive at day 100
3	Acetate buffer	600 rads	6/6 dead at day 23
4	Neuraminidase	600 rads	6/6 dead at day 25
5	Neuraminidase	300 rads	1 dead at day 22 2 alive at day 60
6	Neuraminidase	150 rads	1 dead at day 21 2 alive at day 60
		Rechallenge experiments	
7	Nil	40,000 neuraminidase treated *L* 1210 cells 30 days previously	6/6 alive at day 60
8	Nil	Nil	3/3 dead at day 21

In the inoculation experiments each mouse received intraperitoneally 40,000 *L* 1210 cells in 0·5 ml. of medium 199. In the rechallenge experiments each mouse received untreated *L* 1210 cells 30 days after primary inoculation. Total body irradiation was from a source of cobalt-60 placed 65 cm from the skin; "build-up" was provided by a 'Perspex' sheet 4 mm thick placed immediately above the mice, and was completed 2 h before inoculation.

Results are shown in Table 1. All mice which received 40,000 untreated tumour cells died within 20 days, but mice which received tumour cells which had been treated with neuraminidase have remained in apparent normal health for more than 100 days. The viability of the treated cells was demonstrated *in vivo* by their growth in irradiated recipients. Such recipients of both treated and untreated cells died with haemorrhagic ascites and splenomegaly; ascitic fluid removed before death contained large numbers of cells with the morphological characteristics of *L* 1210 cells and which caused fatal haemorrhagic ascites on passage into new recipients.

The growth of the neuraminidase-treated *L* 1210 cells in irradiated recipients but not in intact recipients strongly suggests that an intact immune system is necessary to prevent their growth. Evidence that untreated mice which had received treated cells were immune was provided by their survival after challenge with untreated tumour cells. Susceptible mice are known to die after inoculation with one *L* 1210 cell[4] and an estimated dose of 100 cells, the smallest used in this laboratory, has always proved fatal in the strain of mice used in these experiments. Animals which received a challenge dose of 8,000 untreated tumour cells have survived more than 60 days; recipients of 40,000, 200,000 and 1,000,000 *L* 1210 cells died within 30 days. The detection of immunity in these mice was therefore dose dependent. Sera from tumour-bearing mice did not agglutinate *L* 1210 cells in slide agglutination tests when diluted more than 1/8. Sera from two immune mice agglutinated *L* 1210 cells at a maximum dilution of 1/1,024.

The action of neuraminidase is to liberate sialic (*N*-acetylneuraminic) acid moieties by cleaving their O-glycoside links with underlying amino-sugars[5]. The development of immunity after exposure to neuraminidase-treated tumour cells is therefore consistent with the concept that sialic acid groups in the cell periphery can mask cell surface antigens from detection by the immune system. It remains to be determined whether the masking is effected by electrostatic effects, by steric hindrance or by some other mechanism. These results are also consistent with findings reported from this laboratory on murine trophoblast and the Landschutz ascites tumour, the observations of Lindenmann and Klein[6] with Ehrlich's ascites tumour cells injected along with neuraminidase and those of Sanford[7] with neuraminidase-treated *TA3* cells. The masking of antigens may be a significant factor in oncogenesis and tumour host interaction.

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