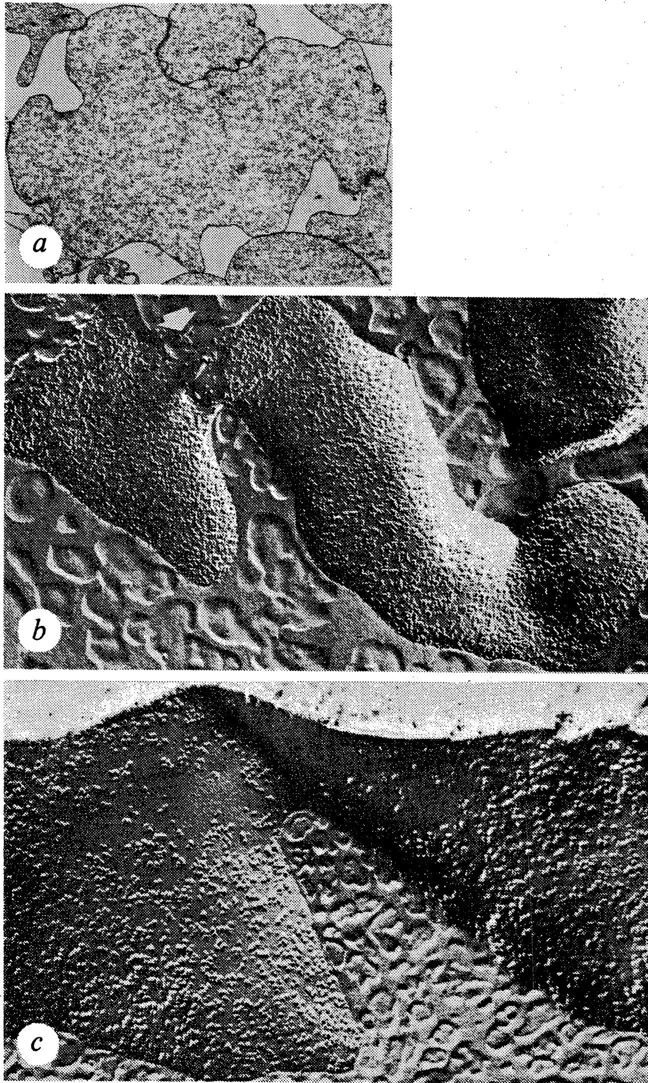

Fig. 1 Electron micrographs of human erythrocyte ghosts in the process of fusing. Human erythrocyte ghosts were prepared and fused as described in the legend to Table 1, experiment (1). The bivalent metal used was $CaCl_2$ (2 mM). Samples were incubated for 25 min at 37 °C for fusion. Preparation of sections for electron microscopy (*a*) was as described before[27]. Freeze-fractured cells were prepared essentially as described before[20] with the following modifications. Erythrocyte ghosts were fixed immediately after fusion by addition of glutaraldehyde (Ladd Research Industries) to a final concentration of 1% (v/v) and centrifuged at 12,000*g* for 5 min. *a*, Electron micrograph of a polyghost tightly agglutinated with unfused ghosts (× 4,800). *b*, Freeze-fracture picture of two cells tightly agglutinated befoie fusion. Interdigitated region of contact (arrow) is poor in intramembrane particles (× 38,280). *c*, Freeze-fracture picture of fused ghosts. Note folded smooth area separating surfaces with tightly packed intramembrane particles. The smooth area is presumed to be a region of fusion between two ghosts (× 61,200).

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NEHAMA ZAKAI
RICHARD G. KULKA
ABRAHAM LOYTER

Department of Biological Chemistry,
Hebrew University of Jerusalem,
Jerusalem, Israel

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T lymphocytes with promiscuous cytotoxicity

CYTOTOXIC T lymphocytes generated during a unidirectional mixed lymphocyte culture (MLC) lyse target cells which have the antigenic phenotype of the allogeneic stimulating cells. The cytotoxic effect is restricted to cells that bear the same major histocompatibility antigens (H–2 antigens in mice) as the stimulating cells[1]. H–2 restriction of cytotoxicity is also seen when immune T lymphocytes react *in vitro* to cells bearing viral[2], chemical[3], or minor histocompatibility antigens[4] or following autosensitisation against unmodified fibroblasts[5]. Thus, H–2 restriction of cytotoxic lymphocytes has been observed in many circumstances. We now report that cultures of normal spleen cells generate T lymphocytes that damage target cells regardless of their H–2 phenotype.

To investigate the influence of alloantigens on the generation of cytotoxic T lymphocytes, we cultured normal mouse spleen in the presence or absence of allogeneic stimulator spleen cells or fibroblasts. After 5 d culture, the responder lymphocytes were tested against various fibroblast monolayer targets using a modification of a standard microcytotoxicity assay. In this system the number of adherent fibroblasts remaining after 48 h of incubation with the cultured spleen cells was measured by uptake of radioactive ^{51}Cr. As seen in Table 1*a*, C57L ($H–2^b$) spleen cells, after incubation with irradiated CBA stimulator cells ($H–2^k$), caused fourfold greater cytotoxicity of **B10.Br** ($H–2^k$) than C57BL/6 ($H–2^b$) fibroblasts. This demonstrated the expected relative H–2 restriction of T-cell cytotoxicity which results from an allogeneic MLC. The presence of allogeneic fibroblasts also induced H–2-restricted cytotoxicity (Table 1*b*).

In contrast, spleen cells cultured alone, without allogeneic stimulator cells, showed a similar high degree of cytotoxicity against target fibroblasts without H–2 restriction (Table 1*c*). In each of 20 experiments, normal spleen cells cultured for 5 d developed promiscuous cytotoxicity that did not discriminate among the H–2 phenotypes of target fibroblasts.

To identify the effector cell in the population of cultured spleen cells, the influence of anti-θ treatment[6] was tested with the results shown in Table 2. The marked decrease of cytotoxic activity after anti-$\theta + C'$ treatment suggests that a T lymphocyte was necessary for the expression of promiscuous cytotoxicity. This conclusion was supported by the finding that the cytotoxic effect was not decreased by filtering the effector cells through nylon wool. Thus, promiscuous cytotoxicity, like H–2 restricted cytotoxicity[7], seems to be a function of T lymphocytes. Cell-free medium

in which effector lymphocytes had been cultured for 48 h showed no cytotoxicity against target fibroblasts when compared with fresh medium. Therefore there was no evidence for a nonspecific cytotoxic factor produced by the lymphocytes.

Experiments were carried out to identify the requirements for induction of promiscuous cytotoxicity. Fresh spleen cells or spleen cells cultured for up to 48 h did not produce this effect. Cells cultured for 3 d had 50% of maximal cytotoxic activity. Peak activity was usually attained after 4–5 d of culture and persisted undiminished for up to 9 d.

Table 1 Induction of H-2-restricted or promiscuous cytotoxicity by culture of normal spleen cells

Induction culture*				Target fibroblasts		
Spleen cells		Allogeneic stimulator cells				
Strain	H–2	Strain	H–2	Strain	H–2	% Cytotoxicity†
a C57L	b	CBA spleen cells	k	C57L	b	27
				B10.BR	k	98
b B10	b	B10A fibroblasts	a	B10	b	3
				B10.A	a	83
B10.A	a	B10 fibroblasts	b	B10.A	a	8
				B10	b	84
				B10.D2	d	7
				B10.BR	k	5
c C57L	b	None		C57L	b	75
				B10.BR	k	67
B10	b	None		B10	b	92
				B10.D2	d	91
				B10.BR	k	89
				B10.A	a	96
B10.A	a	None		B10.A	a	93
				B10	b	94
				B10.D2	d	94
				B10.BR	k	94
NZB	d	None		NZB	d	78
				B10.A	a	73
C57BL/6	b	None		C57BL/6	b	76
				NZB	d	81
BALB/c	d	None		DBA/2	d	90
				C57BL/6	b	92

*Suspension of spleen cells of 6–8-week-old male mice were prepared by forcing fragments of spleen through a tantalum gauze mesh. The spleen cells were suspended in medium consisting of RPMI 1640 (Grand Island, Gibco) plus 10% foetal calf serum (FCS) (heat inactivated at 56% for 30 min; Gibco), 5×10^{-5} M 2-mercaptoethanol, sodium pyruvate 1 mM (Microbiological Associates), non-essential amino acids 0.1 mM (Microbiological Associates) and penicillin (5,000 units ml^{-1}) and streptomycin (5,000 μg ml^{-1}) (RPMI+10% FCS). The spleen cells were washed, centrifuged and resuspended at a concentration of 7×10^6 viable nucleated cells in 2 ml of RPMI+10% FCS. The spleen cells were incubated for 5 d in 16-mm tissue culture wells (Costar) in a volume of 2 ml per well at 37 °C in moist air plus 10% CO_2. After induction the spleen cells were collected from the culture wells by repeated pipetting of the medium, washed by centrifugation and tested for cytotoxicity. Some induction cultures contained allogeneic stimulator cells which were either 3.5×10^6 irradiated (2,000 r.) spleen cells (*a*), or monolayers of 10^5 fibroblasts (*b*). The fibroblasts were prepared from 13–17-d-old mouse embryos as described[15].

†Cytotoxicity was assayed in quadruplicate by incubating test spleen cells with target fibroblasts at effector–target ratios of either 50:1 or 100:1. Control cultures contained fresh uncultured spleen cells of the same genotypes as the test spleen cells. One million spleen cells in 2 ml RPMI+10% FCS were added to either 10^4 or 2×10^4 target fibroblasts which had been cultured for one day in 16-mm culture wells. After 44 h, the spleen cells and detached fibroblasts were aspirated and the adherent fibroblasts washed four times with RPMI×10% FCS. To measure the relative numbers of adherent fibroblasts, we incubated each culture well for 40 min with 3 μCi ^{51}Cr in 0.2 ml of 0.3 M sucrose. The wells were then washed twice with RPMI+10% FCS and aspirated dry. Uptake of ^{51}Cr was determined by digesting the fibroblasts with 1 ml 0.1 N NaOH. After 30 min the contents of each well were transferred to plastic tubes and radioactivity was measured in gamma counter. Percentage cytotoxicity was computed as (1–mean c.p.m. (target with test spleen cells/target with control fresh spleen cells)×100). The standard deviations were always less than 10% of the mean c.p.m.

Adherent cells such as macrophages are involved in the induction of cytotoxic T lymphocytes by allogeneic cells[8]. The role of an adherent population in the induction and effector phases of promiscuous cytotoxicity was investigated with the results shown in Table 3. Spleen cells were plated on Petri dishes for 1 h at 37 °C. The non-adherent cells were removed, cultured alone for 5 d and then assayed for cytotoxicity. The non-adherent cells had a markedly decreased capacity to generate cytotoxic cells when compared with an unseparated population. Conversely, absorption of spleen cells on Petri dishes after 5 d of culture did not reduce their capacity to mediate cytotoxicity. Fractionation of spleen cells on nylon wool columns before or after *in vitro* culture confirmed the requirement for an adherent cell in the induction of cytotoxicity, but not in the effector phase.

The inducer of promiscuous cytotoxicity is unknown. Foetal calf serum in the culture medium might have contained mitogen-like substances capable of inducing polyclonal differentiation of effector lymphocytes[9]. It is also possible that the foetal calf serum was specifically immunogenic during induction. Its association with the fibroblasts in the target cultures could therefore have triggered cytotoxicity to the fibroblasts[10]. This is unlikely, however, since we found that adding either allogeneic or syngeneic fibroblasts to the inducing cultures led to H–2 restriction of the resulting cytotoxicity (our unpublished results).

Table 2 Effect of anti-θ treatment on promiscuous cytotoxicity

Treatment of C57BL/6 cytotoxic spleen cells	% Cytotoxicity of C57BL/6 fibroblast targets
None	94
C′	91
Anti-θ	93
Anti-θ+C′	33

Promiscuous cytotoxicity was induced and tested as in Table 1. After induction, the spleen cells were untreated, or treated with complement (C) anti-θ globulin or anti-θ globulin+C′ and tested for cytotoxicity against syngeneic fibroblasts (see Table 1). Anti-θ globulin (Cohn Fr. II of AKR anti C3Hθ serum) was added to spleen cells (30×10^6–50×10^6) at a dilution of 1:10 in 0.3 ml and incubated at room temperature for 45 min. C′ (fresh guinea pig serum absorbed with C57BL/6 spleen cells for one hour at 0 °C) was used at a final dilution of 1:9 in 1 ml and incubated at 37 °C for 1 h. Some spleen cells were treated sequentially with anti-θ globulin followed by C′.

Regardless of the underlying mechanism, it is clear that *in vitro* culture of spleen cells can lead to the generation of T lymphocytes with relatively unrestricted cytotoxicity. How then does addition of allogeneic cells to the culture abort promiscuous cytotoxicity? It is possible that the relevant alloantigens may favour the selective proliferation of clones. Alternatively, the induction of specific cytotoxicity by allogeneic cells may be accompanied by the active suppression of unselected clones.

The emergence of nonspecific suppressor activity following *in vitro* culture of normal spleen cells has been observed by Hodes and Hathcock[11] and by Burns *et al.*[12]. "Precultured" spleen cells could not be induced into cytotoxic effectors in an MLC. Such cells also suppressed the responses of fresh spleen cells to allogeneic or modified syngeneic targets[11]. The induction of antibody production *in vitro* was also inhibited by spleen cells cultured in a similar manner[12]. This suppression was mediated by a T lymphocyte and had similar kinetics and cellular requirements for induction as the promiscuous cytotoxicity reported here.

What is the relationship between promiscuous cytotoxicity and the reports of suppressor activity that developed spontaneously in cultures of mouse spleen cells? These

Table 3 Effect of removal of adherent cells on promiscuous cytotoxicity

Absorption of BALB/c spleen cells on Petri dishes	% Cytotoxicity of NZB fibroblast targets
None	76
Before induction	18
After induction	70

Promiscuous cytotoxicity was induced and tested as in Table 1. Some of the spleen cells were absorbed before induction by placing 20×10^6 cells in 3 ml RPMI+10% FCS in 60-mm tissue culture dishes (Falcon) and incubating at 37 °C for 1 h. Non-adherent cells were removed by repeated pipetting, cultured for 5 d and tested for cytotoxicity. Some spleen cells were cultured for 5 d and then absorbed as above. The non-adherent cells were then tested for cytotoxicity.

phenomena conceivably result from activities of distinct subclasses of T lymphocytes: cytotoxic and suppressor cells. Alternatively, these phenomena may reflect the dual function of a single subclass of lymphocytes. In other words, these different *in vitro* measurements may be manifestations of the same biological function. Suppression of an MLC or antibody production[11,12] could result from indiscriminate damage to the reacting lymphocytes. And the effect we observed may be yet another manifestation of suppressor cell function. Identity of suppressor and cytotoxic T lymphocytes is also supported by the finding that the cells mediating these activities share the same Ly-2,3 T-cell marker[13,14].

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CHAIM SHUSTIK
IRUN R. COHEN*
ROBERT S. SCHWARTZ
ELIZABETH LATHAM-GRIFFIN

Hematology Service,
New England Medical Center Hospital,
Boston, Massachusetts 02111

SAMUEL D. WAKSAL

Immunology Branch,
National Cancer Institute,
National Institutes of Health,
Bethesda, Maryland 20014

Received July 15; accepted August 30, 1976.

*Present Address: Department of Cell Biology, The Weizmann Institute of Science, Rehovot, Israel.

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