CYTOTOXIC POTENTIAL OF STIMULATED HUMAN LYMPHOCYTES*

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Lymphocytes from unsensitized animals or humans damage allogeneic cells in tissue culture when aggregated to the latter by means of phytohemagglutinin (PHA) (1–3). Under the experimental conditions used, injury of the target cells was not seen or was slight in the absence of PHA. When the PHA-treated lymphocytes were separated from the target cells by a Millipore filter, cell damage was prevented (1). Moreover, extracts from PHA-treated cells did not damage the tissue culture cells (1). Therefore, close contact between living lymphocytes and target cells seemed to be necessary for a cytotoxic reaction. However, PHA is also known to stimulate lymphocytes, leading to transformation into blast cells and mitosis (4, 5). Hence, it could be assumed that a PHA-induced stimulation of lymphocytes could contribute to their cytotoxic action.

The aim of the present investigation was to study the significance of stimulation for the cytotoxic effect of lymphocytes on allogeneic target cells. Since lymphocyte-stimulating and leukoagglutinating factors of PHA have not been separated as yet (6, 7), cytotoxicity and stimulation of lymphocytes were determined in parallel incubations at different concentrations of PHA. Furthermore, the cytotoxic effects of lymphocytes were also tested after prestimulation with a culture filtrate of *Staphylococcus aureus*, with purified protein derivative (PPD), or with allogeneic lymphocytes. Chang cells (human liver), labeled with chromate-^{51}Cr, were used as target cells in all experiments. Injury of these cells was recorded quantitatively by measuring the release of isotope after incubation with lymphocytes (8).

Materials and Methods

Preparation of Lymphocytes.—Healthy volunteers, 20–50 yr of age, served as donors. Lymphocytes were isolated from defibrinated venous blood by sedimentation in gelatin (9). The cells were washed twice in Hanks' solution buffered with 0.15 M tris buffer, pH 7.4, and counted in a Bürker chamber after staining of dead cells with trypan blue. The lymphocytes

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were either used immediately for cytotoxicity experiments or preincubated for varying periods of time as described below. A detailed description of the preparation of lymphocytes has been given elsewhere (8).

Incubation of Lymphocytes.—The lymphocytes were suspended in Eagle's medium (10) with 15% heat-inactivated (30 min at 56°C) human AB serum. The concentration was 1–2 $\times$ 10^6 cells/ml. 10–15 ml of the suspension was added to milk dilution bottles. The flasks were incubated at 37°C in an upright position and gassed continuously with 95% air and 5% carbon dioxide. The stimulant was added at the start or during the course of the preincubation as indicated in the text. After incubation, for various periods of time, the cells were collected by centrifugation at 350 *g* for 10 min and were then washed once in Hanks-tris buffer. For cytotoxicity experiments, the appropriate number of living lymphocytes was added to tubes containing isotope-labeled Chang cells (see below).

Aliquots from each preincubation were smeared on cover slips and stained with MacNeal's tetrachrome. The percentage of transformed lymphocytes was determined by counting 500 cells. In some experiments the DNA synthesis of preincubated lymphocytes was measured. For this purpose, 10^6 lymphocytes were added to sterile centrifuge tubes and were incubated for a further 24 hr in the presence of 0.2 μc thymidine-2-^{14}C (specific activity 15 mc/mmole; Radiochemical Centre, Amersham, England). The cells were washed five times (twice in the presence of an excess of thymidine-^{12}C) and the isotope content of the cell pellet was determined in a gas flow counter with a thin-windowed Geiger-Müller tube as previously described (11).

In the experiments in which cytotoxicity and stimulation of lymphocytes were compared after treatment with different doses of PHA, 10^6 lymphocytes were incubated for 44 hr in Eagle's medium containing 5% human serum. 0.2 μc thymidine-2-^{14}C (specific activity 35 mc/mmole) was then added. After 4-hr incubation with the isotope, incorporation into DNA was determined as described. For determination of RNA synthesis, 10^6 lymphocytes per tube were incubated with the PHA for 22 hr. 0.2 μc uridine-2-^{14}C (specific activity 42 mc/mmole; Radiochemical Centre) was added and incubation was continued for another 2 hr. The tubes were then rapidly cooled in iced water and centrifuged for 5 min at 500 *g*. The pellets were washed four times with ice cold buffer in the presence of an excess of uridine-^{12}C. The radioactivity of the cells was measured as described for thymidine-^{14}C (11). Although trichloroacetic acid (TCA) soluble and TCA-insoluble nucleotides were not separated in the present experiments, the incorporation data can be considered to reflect RNA synthesis (12).

Cytotoxicity Experiments.—The method used here has been described in detail elsewhere (8). Chang cells (human liver) cultivated in spinner cultures were labeled with sodium chromate-^{51}Cr (specific activity 100–300 μc/μg chromium; Radiochemical Centre). The cells were washed and counted after the addition of trypan blue. 5 $\times$ 10^4 or 10^5 living and labeled Chang cells were pipetted into roller tubes and incubated with a 5- to 50-fold excess of lymphocytes. In some of the tubes, lymphocytes and Chang cells were aggregated with PHA (5 μl PHA-M per ml of medium; Difco Laboratories, Inc., Detroit, Mich.). Tubes containing Chang cells only, or Chang cells plus PHA served as controls. The nutrient medium consisted of Parker 199 supplemented with 100 units of penicillin and 100 μg of streptomycin per ml. 5% heat-inactivated fetal calf serum (or in some experiments, 5% human AB serum) was added. Final volume of the incubation mixture was 1.5 ml.

The tubes were loosely fitted with screw caps and incubated for 24 hr at 37°C in an atmosphere of 95% air and 5% carbon dioxide. After incubation, the total radioactivity of the samples was determined in a well type scintillation counter. The tubes were centrifuged and 1.0 ml cell-free medium was removed for counting. The radioactivity of the medium was expressed as percentage of the total radioactivity of the sample and was used as a measure of cell damage. Cytotoxicity was expressed as per cent isotope release in sample − per cent isotope release in lymphocyte-free control (spontaneous release) = corrected release (see

reference 8). The magnitude of spontaneous release in each experiment is indicated in figure legends and tables. All numbers are the means of two independent incubations. The standard error of the means is 2.4% (8).

Determination of Aggregation.—Immediately after the determination of isotope release, the cells were suspended by gentle shaking. Aliquots were examined for mixed aggregation by phase-contrast microscopy. The aggregation between lymphocytes and Chang cells was scored from − to ++++: − designates absence of mixed aggregates; ++++, complete mixed aggregation with no free cells, as usually found in tubes containing 5 μl PHA per ml of medium; +, small mixed aggregates consisting of 1–2 Chang cells and 1–5 lymphocytes; ++, mixed aggregation of intermediate strength; and +++, strong mixed aggregation with only a few free lymphocytes and Chang cells.

Staphylococcus Filtrate.—A crude culture filtrate was prepared from *Staphylococcus aureus* (strain 209, Oxford, obtained from the Bacteriological Laboratory, Karolinska Hospital, Stockholm, Sweden) by the method described by Ling and Husband (13). The bacteria were grown in 500 ml of continuously stirred Parker 199 medium without antibiotics for 4 days. The culture medium was sterilized by filtration through a Seitz filter. The extract was stored at −20°C. The same batch was used in all experiments. For stimulation of lymphocytes, the appropriate dilution of the extract was made with the incubation mixture.

Purified Protein Derivative (PPD) without preservative was obtained from Parke, Davis & Co., Detroit, Mich.

RESULTS

The Influence of the Dose of PHA on Cytotoxicity and Stimulation of Lymphocytes and on Mixed Aggregation.—

Lymphocytes without additions were preincubated for 24 hr at 37°C. The cells were collected by centrifugation and suspended in fresh Eagle's medium with 5% human serum. Cytotoxicity and stimulation of DNA and RNA synthesis were then determined in parallel incubations with the same medium containing varying concentrations of PHA (0.065–64 μl/ml of medium). Cytotoxicity, mixed aggregation and RNA synthesis were measured after 24 hr of incubation, and DNA synthesis after 48 hr.

At concentrations of PHA lower than 0.2 μl/ml, the lymphocytes did not damage the target cells (Fig. 1). Maximal destruction of Chang cells occurred in tubes containing 4–8 μl PHA, while higher concentrations were inhibitory. Mixed aggregation was observed even at very low concentrations of PHA. At 8 μl/ml, all lymphocytes and Chang cells were completely and randomly aggregated. At higher concentrations the microscopical pattern of aggregation was unchanged but the aggregates were bigger.

RNA synthesis was estimated as incorporation of uridine-^{14}C at a time (22–24 hr) before DNA synthesis was initiated. It was only slightly stimulated by 1.0 μl PHA and was optimal at 16 μl PHA. 64 μl PHA was inhibitory. A similar pattern was seen for DNA synthesis which showed a peak at 4.0 μl PHA/ml.

PHA was not toxic to the Chang cells at any concentration. When the survival of lymphocytes was measured after 24 hr by their ability to exclude trypan blue, even high doses of PHA (16 and 64 μl) did not affect their viability.

Cytotoxicity of Lymphocytes Stimulated by Staphylococcus Filtrate.—A filtrate of *Staphylococcus aureus* (SAF) stimulates a large proportion of human lymphocytes to DNA and RNA synthesis and to transformation into blastoid cells (13). The SAF preparation used in this investigation caused maximal blastoid trans-

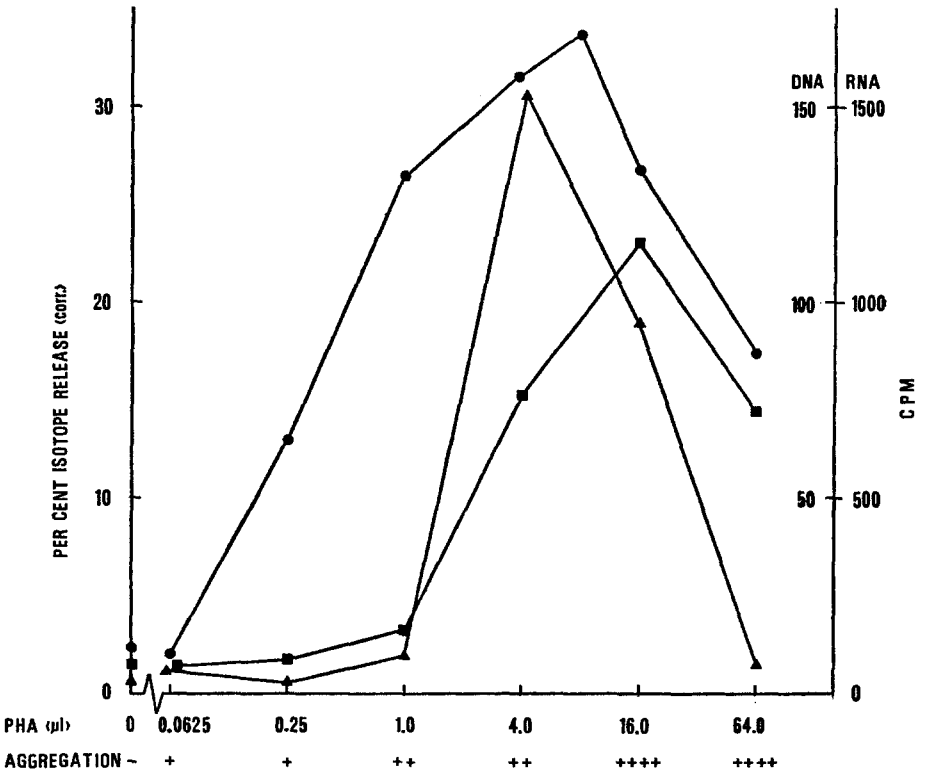

FIG. 1. The influence of the concentration of PHA on cytotoxicity and stimulation of lymphocytes. ●, per cent isotope release from Chang cells incubated with lymphocytes for 24 hr. Numbers corrected for spontaneous release (23.8%). Ratio lymphocytes/Chang cells 25:1. ■, incorporation of uridine-^{14}C into lymphocytes (cpm/culture) measured 24 hr after start of incubation; ▲, incorporation of thymidine-^{14}C into lymphocytes (cpm/culture) measured 48 hr after start of incubation. Each symbol is the mean of two independent incubations.

formation and DNA synthesis at dilutions ranging from 1:15 to 1:75. Higher concentrations were slightly inhibitory. The maximal incorporation of thymidine-^{14}C induced by SAF was about 50% of that seen in PHA-stimulated cells.

The staphylococcal filtrate is practically devoid of leukoagglutinating activity. Therefore, it was considered suitable as a substitute for PHA in the cytotoxicity test. In a first series of experiments, SAF was added to mixtures of

lymphocytes and labeled Chang cells. Damage of the latter was determined after 24 hr of incubation and compared with that induced by PHA (Table I). In most experiments, some SAF-induced cell damage was observed when lymphocytes were present. However, this was much weaker than that seen in

TABLE I

Cytotoxicity of Lymphocytes in the Presence of Staphylococcus Filtrate (SAF)

Experiment No.	Incubation	Isotope release (corr.)	Mixed aggregation
		%	
I	SAF	−0.5*	
	Lymphocytes	6.9	−
	Lymphocytes + SAF	17.1	+
	Lymphocytes + PHA	36.9	++++
II	SAF	−1.7*	
	Lymphocytes	3.6	±
	Lymphocytes + SAF	9.9	±
	Lymphocytes + PHA	21.2	++++

Final dilution of SAF 1:15. Lymphocyte/Chang cell ratio 25:1. Spontaneous isotope release in Experiment I, 30.5%, in Experiment II, 30.6%.

* Lower than spontaneous release.

TABLE II

Cytotoxicity of Lymphocytes on Chang Cells Pretreated with Staphylococcus Filtrate

Incubation	Isotope release (corr.)	
	Untreated	Pretreated
	%	%
PHA	−1.4*	5.4
Lymphocytes	6.9	7.7
Lymphocytes + PHA	30.2	29.7

Lymphocyte/Chang cell ratio 25:1. The spontaneous isotope release from untreated Chang cells was 22.3% and that of Chang cells treated with staphylococcus filtrate was 21.8%. Time of incubation, 24 hr.

* Lower than spontaneous release.

the presence of PHA and lymphocytes. Under these conditions, only a weak mixed aggregation or none at all was brought about by SAF. SAF was not toxic for the Chang cells.

The possibility that some factor in the staphylococcal filtrate binds to the Chang cells and makes them susceptible to the cell damaging activity of lymphocytes was tested in an experiment, the results of which are shown in Table II. 2×10^6 living and labeled Chang cells were incubated for 1 hr at

37°C with 3 ml SAF at a final dilution of 1:3. Control Chang cells were incubated under the same conditions without SAF. These cells were washed twice and used as target cells. SAF treatment of the Chang cells did not alter their sensitivity to the cytotoxic action of lymphocytes.

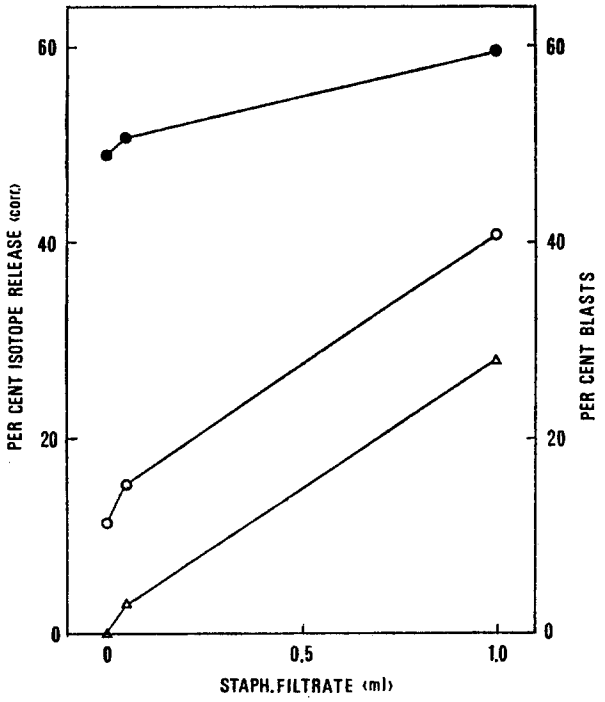

FIG. 2. Cytotoxicity of lymphocytes pretreated with different concentrations of staphylococcus filtrate. The lymphocytes were preincubated for 72 hr in 15 ml of medium containing the volume of staphylococcus filtrate indicated on the abscissa. Ratio lymphocytes/Chang cells 25:1. Time of incubation 24 hr. Spontaneous isotope release 23.0%. ●, corrected isotope release, lymphocytes + PHA; ○, corrected isotope release, lymphocytes; △, blastoid transformation.

For further study of the possible relationship between stimulation and cytotoxicity, lymphocytes were treated with SAF prior to incubation with Chang cells. Fig. 2 gives the results of a typical experiment in which lymphocytes were preincubated for 3 days with two different doses of SAF. After pretreatment with SAF at a final dilution of 1:15, 28% of the lymphocytes were transformed. Cells pretreated in this manner were highly cytotoxic for Chang cells even in the absence of PHA. The damage of the Chang cells was significantly elevated over that obtained with lymphocytes preincubated for 3 days without SAF. At the lower dose (final dilution 1:300) SAF caused the transformation of 3% of

the lymphocytes. This preparation was also slightly more cytotoxic than the control. In all PHA-free incubations, mixed aggregation was scored as +. As also seen in Fig. 2, SAF-treated lymphocytes were more cytotoxic than untreated lymphocytes when incubated with Chang cells in the presence of PHA.

The effect of the duration of prestimulation is shown by the typical results recorded in Fig. 3. SAF (final dilution 1:15) was added to the lymphocytes at 72, 48, 24, or 0.5 hr before the start of the cytotoxicity experiment. Lympho-

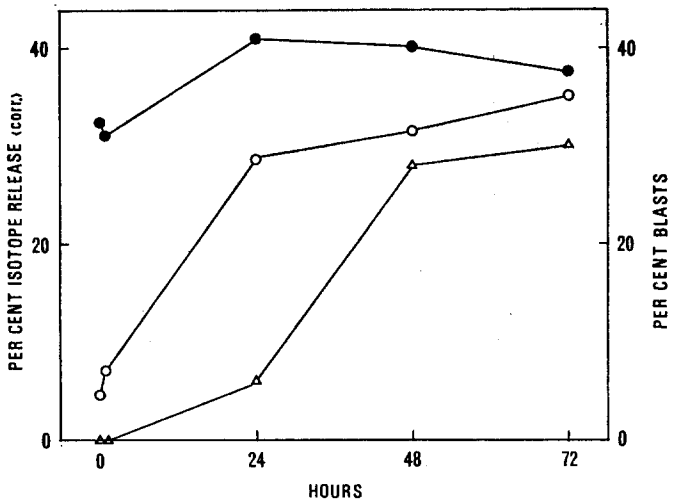

FIG. 3. Cytotoxicity of lymphocytes pretreated with staphylococcus filtrate. The effect of time of stimulation. Final dilution of staphylococcus filtrate 1:15. Lymphocyte/Chang cell ratio 25:1. Time of incubation 24 hr. Spontaneous isotope release 21.5%. For explanation of symbols see Fig. 2.

cytes prestimulated with SAF from 24 to 72 hr were highly cytotoxic. The maximal damage of the Chang cells in these tubes was comparable to that induced by unstimulated cells in the presence of PHA. The latter also slightly potentiated the effect of SAF-prestimulated lymphocytes. In the PHA-free tubes, control lymphocytes gave a mixed aggregation scored as +, while that obtained with SAF-treated lymphocytes was scored as ++.

Fig. 3 also shows that a slight damage to the Chang cells was induced by lymphocytes preincubated with SAF for only 0.5 hr. A more detailed study of the cytotoxic effects of lymphocytes exposed to SAF for short periods is seen in Fig. 4. In this experiment, lymphocytes were pretreated with SAF at a final dilution of 1:30 for 3, 6, 18, or 27 hr. Although the cell-damaging effect of these cells was weaker than in the preceding experiment, it increased significantly with time of SAF treatment.

Prestimulation with PPD.—Lymphocytes from tuberculin-positive and tuberculin-negative donors were incubated with PPD for various periods of time. 2.5 μg of PPD per ml of medium gave optimal synthesis of DNA and blastoid transformation. This concentration was used in all preincubation experiments.

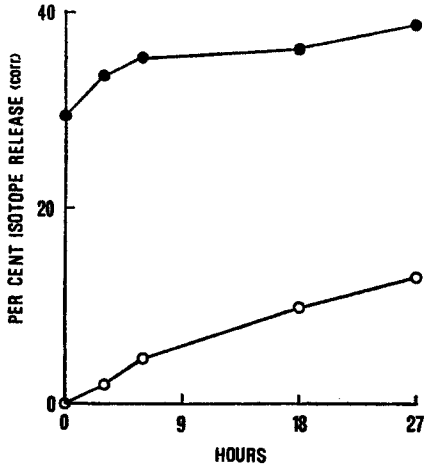

FIG. 4. Cytotoxicity of lymphocytes pretreated with staphylococcus filtrate, short term preincubation. Final dilution of staphylococcus extract 1:30. Lymphocyte/Chang cell ratio 25:1. Time of incubation 24 hr. Spontaneous isotope release 24.5%. For explanation of symbols see Fig. 2.

Table III gives the results of three experiments performed on different occasions. In Experiment I, lymphocytes of the strongly tuberculin-positive donor G. H. were treated with PPD for 5 days, at which time 41% of the cells were transformed into blasts. PPD-transformed lymphocytes were highly cytotoxic and cell damage was only slightly increased by PHA. In tubes without PHA the mixed aggregation of PPD-treated cells and Chang cells was scored as +. PHA induced a ++++ aggregation as usual.

In Experiments II and III, the cytotoxic effects of G. H. lymphocytes were compared with those of a donor, F. H., who was tuberculin negative. In both experiments, PPD-pretreated G. H. lymphocytes were cytotoxic even in the absence of PHA, while untreated lymphocytes were inactive. The relatively low effect noted in Experiment III may be due to the lower lymphocyte/Chang cell ratio used in this experiment (8). In contrast, F. H. lymphocytes, which also were inactive when untreated, were only slightly cytotoxic after pretreatment with PPD under identical conditions. The table also shows that the rise in cytotoxic potency was correlated with the differential response of these lymphocytes to PPD when recorded either as per cent blast transformation or

as stimulation of DNA synthesis. PHA potentiated the cytotoxic efficiency of the lymphocytes in all cases, but a difference in activity between PPD-treated samples of G. H. and F. H. origin was maintained.

TABLE III

Cytotoxicity of Lymphocytes Pretreated with PPD

Experiment No.	Time of preincubation	Donors	PPD	Blasts	DNA synthesis	Lymphocyte/Chang cell ratio	Isotope release (corr.)	
							Without PHA	With PHA
	days			%	*cpm/culture*		%	%
I	5	G. H.	−	0	n.d.*	25:1	−4.7‡	20.2
			+	41	"		38.2	43.2
II	4	G. H.	−	0	n.d.*	15:1	2.5	44.1
			+	36	"		47.6	65.2
		F. H.	−	<1	"		5.4	33.5
			+	<1	"		14.6	50.7
		G. H. + F. H.§	−	3	"		8.9	38.9
			+	14	"		29.6	58.8
III	7	G. H.	−	n.d.*	11	10:1	0.0	24.3
			+	"	211		8.1	44.6
		F. H.	−	"	5		1.0	17.1
			+	"	53		4.2	26.5
		G. H. + F. H.‖	−	"	160		2.1	26.7
			+	"	467		10.8	41.9

Lymphocytes from the tuberculin-positive donor G. H. and the tuberculin-negative donor F. H. preincubated separately or mixed with or without PPD (2.5 μg/ml). Spontaneous isotope release in Experiment I, 45.6%; in Experiment II, 26.2%; in Experiment III, 27.2%.

* Not done.

‡ Lower than the spontaneous release.

§ G. H.:F. H. = 1:9.

‖ G. H.:F. H. = 1:1.

In Experiment II, the effect of mixing the responsive G. H. lymphocytes with a 9-fold excess of unresponsive F. H. lymphocytes was also studied. Both the cytotoxic activity and the blast transformation of the mixed and PPD-treated sample in Experiment II were larger than expected on the basis of strict proportionality. A certain effect was also obtained with the mixed sample which was not pretreated with PPD. The same was true in a similar incubation in

Experiment III, in which G. H. and F. H. lymphocytes were mixed in proportion 1:1 (Table III). The data suggest that cytotoxic activity as well as stimulation in these cases are the expression of an additive effect of PPD-treatment and mixing of histoincompatible lymphocytes (14, 15).

Mixed Cultures.—The observations described in the preceding paragraph

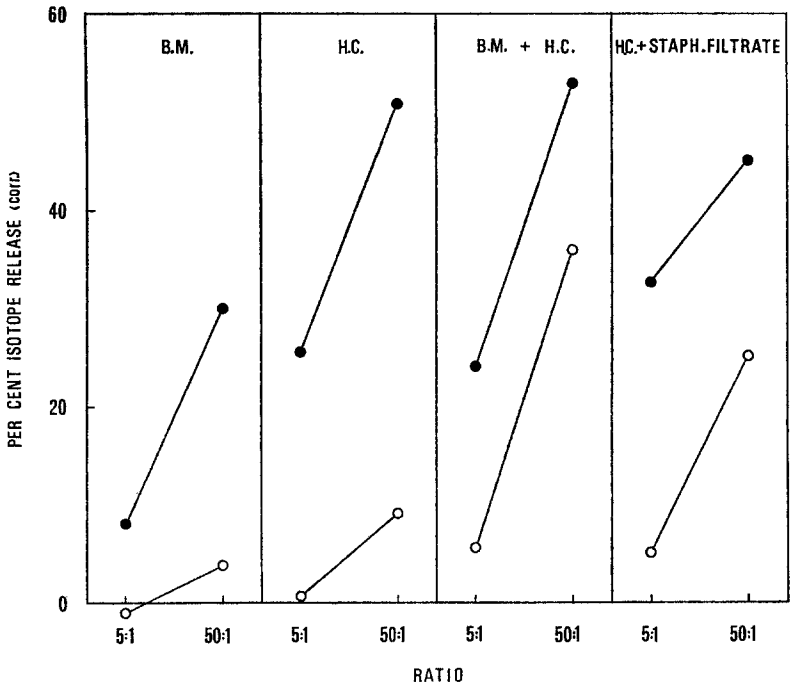

FIG. 5. Cytotoxicity of lymphocytes prestimulated by allogeneic lymphocytes. Lymphocytes from donor B.M. and donor H.C., preincubated for 6 days, either separately or mixed 1:1. H.C. lymphocytes also preincubated with staphylococcus filtrate, diluted 1:20. Spontaneous isotope release 27.7%. For explanation of symbols see Fig. 2.

justified further experiments, in which lymphocyte stimulation was brought about by mixing cells from two histoincompatible donors. In the experiment of Fig. 5, lymphocytes from two donors were kept in culture for 6 days and were then added to Chang cells at two different lymphocyte/Chang cell ratios. When lymphocytes from donors B. M. and H. C. were mixed in equal parts, 45% of the cells were transformed after 6 days. Such cells damaged Chang cells extensively. Cytotoxicity was comparable to that obtained with SAF-pretreated lymphocytes from donor C. R. This sample contained 42% blast cells. Addition of PHA potentiated the effect in all cases. The figure also shows the importance of the lymphocyte/target cell ratio described earlier (8).

In Fig. 6, B. M. and S. G. lymphocytes were preincubated for 6 days separately or in mixtures 1:4 (2% blasts), 1:1 (6% blasts), and 4:1 (3% blasts). As can be seen, the cytotoxicity of these preparations was related to the degree of transformation.

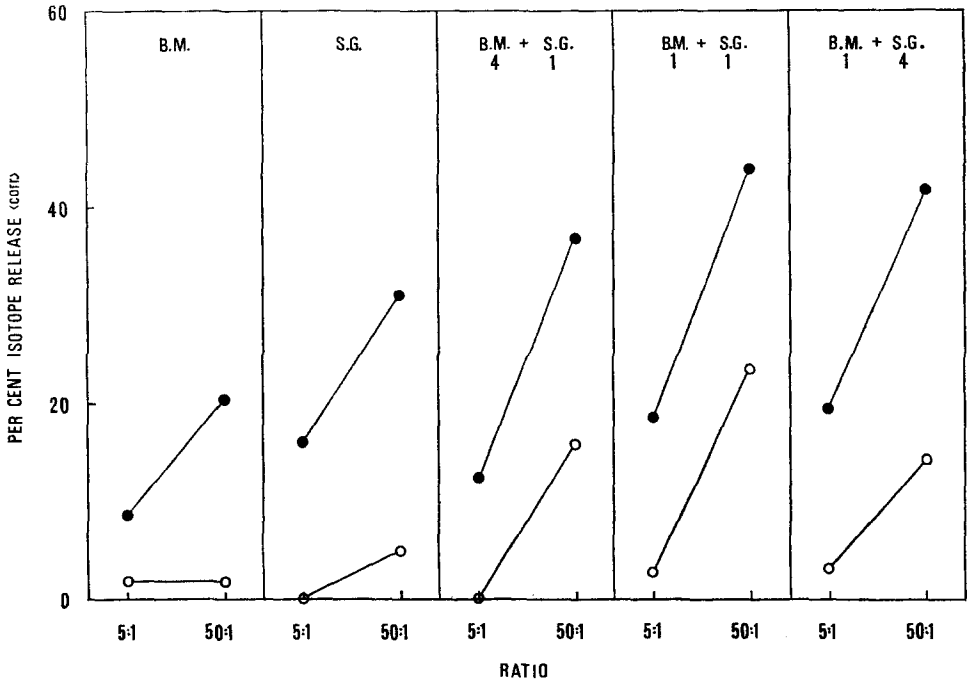

FIG. 6. Cytotoxicity of lymphocytes prestimulated by allogeneic lymphocytes. Lymphocytes from donor B.M. and S.G. preincubated for 6 days separately or mixed in the proportion 4:1, 1:1, and 1:4. Spontaneous isotope release 34.6%. For explanation of symbols see Fig. 2.

DISCUSSION

Stimulation of normal human lymphocytes strongly promotes their cytotoxic action on allogeneic tissue culture cells in vitro. This is suggested by two independent lines of evidence. When cytotoxicity was determined as a function of PHA dose, high concentrations were inhibitory, in spite of the fact that aggregation of the cell types was increased. Optimal cytotoxic effects were obtained at intermediate doses which coincided reasonably well with doses inducing maximal stimulation of DNA and RNA synthesis. The dose dependency of stimulation was similar to that recently reported by others (12, 16, 17). The reason for the inhibitory effect of high PHA doses is unknown.

While the optimal PHA concentrations for all three responses were similar,

some discrepancies were seen at low concentrations. Thus, at PHA doses up to 1 μl/ml, stimulation of uridine-^{14}C incorporation into lymphocytes was insignificant while cytotoxicity was high. Although the rate of uptake of a radioactive metabolite may account for this difference, the data probably imply that cytotoxicity and RNA synthesis are independent expressions of stimulation. This explanation is most likely valid for cytotoxicity and DNA synthesis. The latter had a dose dependency similar to that of RNA synthesis, but is not even initiated within the first 24 hr of incubation (12) when cytotoxicity was measured. Whether, in PHA-stimulated lymphocytes, some early metabolic changes or a change in surface structure is responsible for their cytotoxic activity is a question that requires further study.

The importance of stimulation for lymphocyte cytotoxicity was also shown by applying a variety of nonagglutinating stimulants (SAF, PPD, mixed lymphocyte culture). When lymphocytes treated in this way were added to Chang cells, or were added to them in the presence of the stimulant, mixed aggregation of the cell types did not occur or was slight. Nevertheless, such lymphocytes were cytotoxic. Their reactivity was independent of the kind of stimulant applied but was well correlated to the degree of stimulation recorded as transformation (or DNA synthesis) in parallel incubations. Application of two entirely different stimulants at the same time had additive effects (18). Addition of PHA to the lymphocyte/Chang cell mixture also potentiated the effect of the other agents.

These results are not to be taken to mean that contact of lymphocytes and target cells is without importance in this cytotoxic system. Prevention of cell to cell contacts has previously been seen to inhibit the reaction, and no cytotoxic factor has so far been found in the medium recovered from lymphocytes pretreated with PHA or other stimulants (reference 1 and unpublished data). The experimental conditions applied here allowed for unrestricted contacts between lymphocytes and Chang cells. Stimulated lymphocytes may have surface properties which favor their cytotoxic reactivity. On the other hand, mixed aggregation may itself facilitate stimulation of lymphocytes by foreign antigens on the target cells and may contribute to a cytotoxic reaction. Experiments with certain nonstimulating but aggregating agents would seem to favor this supposition.[1]

The mechanism by which Chang cell injury was effectuated by stimulated lymphocytes is unknown. There is no reason to believe that it involved antibody formation against antigens of the tissue culture cells. PHA and SAF are both considered to constitute general stimulants which bypass the antigen-dependent step and affect the majority of lymphocytes in human peripheral blood (19, 20). They could stimulate a fraction of the lymphocytes to produce anti-

[1] Holm, G. Data to be published.

bodies against the Chang cells. There is no evidence for this assumption. PPD only stimulates lymphocytes from tuberculin-positive donors who have previously been in contact with mycobacterial antigens (19, 21). Stimulation is thus strictly antigen specific. This is also true of mixed lymphocyte cultures where stimulation requires histoincompatibility of the lymphocyte donors (14, 15). The latter system differs from the PPD system in that no previous contact with the stimulating antigens is required for stimulation. Regardless of whether this difference is more apparent than real (18, 22), both systems produce transformed lymphocytes which are highly cytotoxic for Chang cells. The explanation of this phenomenon is hampered by our lack of knowledge of the immunological significance of lymphocyte stimulation by antigen in vitro. In some of the present experiments, nearly 50% of the lymphocytes became transformed within 6 days of incubation with PPD or in mixed culture. Making the not very probable assumption that all transformed cells were committed to produce antibody against the stimulating antigens would imply that they would not also make antibody against Chang cell antigens, unless stimulating antigens and Chang cells had some cross-reacting determinants. In that case, a fraction of the lymphocytes could have produced antibodies able to react with Chang cell antigens as well. Alternatively, stimulation may not automatically lead to antibody production against the stimulating antigens. Antibody formation against these or other antigens might then be induced during or after transformation. Hence, a fraction of the transformed lymphocytes might be stimulated to mount an immune response when exposed to new antigens of the Chang cells. There is no evidence to support this.

Although none of these explanations has as yet been rigorously excluded, the present findings are more compatible with the assumption that cell injury was not produced by antibodies against Chang cell antigens. Rather, cytotoxicity may represent an immunologically nonspecific lymphocyte activity enhanced by stimulation. However, it is also known that the in vitro cytotoxicity of lymphocytes discriminates between target cells of different origin. Cytotoxicity is pronounced when lymphocytes and tissue culture cells are different with regard to histocompatibility antigens (2, 3). Recent findings suggest that target cell antigens other than histocompatibility factors may be involved as well, although higher lymphocyte concentrations and longer incubations may be needed (23, 24). Thus, the cytotoxic reaction also seems to include an immunologically specific recognition step whose nature and limits are as yet poorly defined. The relative importance of the specific recognition mechanism and the presumably unspecific effector mechanism for the outcome of the cytotoxic reaction require further elucidation. The situation is further complicated by the fact that variables, such as lymphocyte concentration, time of incubation, and the nature or physiological state of the target cells will also influence the results.

Chang cells were not damaged when exposed to nonviable human lympho-

cytes, red blood cells, or other tissue culture cells together with PHA under conditions in which viable lymphocytes had full effect (25). Negative results were also obtained with lymphocytes from patients with Hodgkin's disease or chronic lymphatic leukemia (11). Both these cells showed poor response to PHA in vitro and were less cytotoxic for Chang cells than normal lymphocytes. This was recently also found to be valid for lymphocytes from human thymus (26). In all these cases, a relative lack of immunological competence of lymphocytes has been suggested (27–29). PHA- or antigen-induced transformation and proliferation, as well as cytotoxicity, may thus represent related activities of immunologically competent lymphocytes. It is likely that the cytotoxic potential of stimulated lymphocytes plays a role in vivo in connection with host vs. graft and graft vs. host reactions and in the normal lymphocyte transfer test (30). It remains to be seen whether it also contributes to inflammation and tissue damage in autologous in vivo situations, such as delayed hypersensitivity and autoimmune disease.

SUMMARY

Viable and immunologically competent lymphocytes from unsensitized donors damage allogeneic tissue culture cells in the presence of phytohemagglutinin (PHA). This cytotoxicity is specific since syngeneic tissue culture cells are not at all or only slightly damaged under similar experimental conditions. In this investigation, the relation between the stimulation of human lymphocytes and their cytotoxicity was studied. Chang cells (human liver) served as target cells in all experiments. Cell damage was quantitated by measuring the release of isotope from target cells labeled with chromate-^{51}Cr.

The cytotoxicity of the lymphocytes was dependent on the concentration of PHA in the incubation medium. Cell damage was maximal at concentrations of 4–8 μl PHA/ml. Higher concentrations were inhibitory although aggregation was increased and no injury of the lymphocytes was noted. Stimulation of DNA and RNA synthesis in PHA-treated lymphocytes each followed dose response curves which were similar to that of cytotoxicity.

In order to establish whether stimulation without mixed aggregation of lymphocytes and target cells would suffice for cytotoxicity, a series of nonagglutinating stimulants were investigated. Lymphocytes pretreated with a crude filtrate of *Staphylococcus aureus* for periods of 0.5–72 hr damaged Chang cells even in the absence of PHA. Lymphocytes from a tuberculin-positive donor were strongly cytotoxic after prestimulation with PPD while those from a negative donor were inactive. Moreover, strong cytotoxic effects were also obtained with lymphocytes which had been stimulated by preincubation with allogeneic lymphocytes in mixed culture. When two stimulants were applied at the same time, additive cytotoxic effects were seen. Addition of PHA to the lymphocyte/Chang cell mixtures potentiated the cytotoxicity of prestimulated lymphocytes.

The cytotoxic potential of the lymphocytes was in all cases correlated to the degree of stimulation recorded as transformation into blast cells, and was independent both of the degree of aggregation and of the stimulating factor.

These findings are compatible with the assumption that injury of the Chang cells reflected an immunologically nonspecific activity of lymphocytes enhanced by stimulation. The possible importance of this activity for a number of tissue-damaging immune reactions in vivo is pointed out.

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