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Quantitative Studies on Phytohaemagglutinin-Induced Cytotoxicity by Human Lymphocytes against Homologous Cells in Tissue Culture

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Summary. The cytotoxic effect of normal human blood lymphocytes on Chang cells in tissue culture was investigated. Cell damage was measured by release of ^{51}Cr from pre-labelled tissue culture 'target' cells. This method was sensitive and rendered highly reproducible results. Released ^{51}Cr was not re-utilized.

About 25 per cent of the ^{51}Cr was spontaneously released from labelled Chang cells when incubated for 24 hours at 37°. Lymphocytes at a lymphocyte/Chang cell ratio of 25:1 led to a slight increase of this release. When phytohaemagglutinin was also present, about 50–60 per cent of the isotope appeared in the medium. Under these conditions target cells were significantly damaged within 1 hour. At a lymphocyte/Chang cell ratio of only 1:1, weak cytotoxic effects were also noted after 24 hours of incubation. The results of dose–response experiments suggested that a considerable proportion of the lymphocytes participated in the reaction. Individual variation of the cytotoxic effect of lymphocytes from different donors suggested that it could be related to the degree of histoincompatibility between lymphocytes and Chang cells. Under the present conditions contaminating erythrocytes or granulocytes did not interfere with the cytotoxic action of the lymphocytes.

INTRODUCTION

It is now well established that lymphocytes from sensitized animals are able to aggregate *in vitro* to tissue culture cells which carry the specific antigens against which the lymphocyte donor was sensitized. This leads to destruction of the tissue culture cells within 24–48 hours (for references see: Wilson, 1965; Holm, 1966). It has been shown that lymphocytes from unsensitized (normal) donors also were cytotoxic when aggregated to cells in tissue culture by means of phytohaemagglutinin (PHA) (Holm, Perlmann and Werner, 1964). Histoincompatibility between lymphocytes and target cells was a necessary pre-requisite for cell damage. That the damage of the tissue culture 'target' cells was brought about by some activity of living and immunologically competent cells (Holm and Perlmann, 1965) is indicated by: (1) thymocytes and various non-lymphoid cells did not kill allogeneic target cells, and (2) diffusible toxic agents produced by lymphocytes were not detected and extracts of PHA-treated lymphocytes were not cytotoxic.

The results of further studies of the cytotoxic effect of normal human lymphocytes on Chang liver cells in tissue culture will be described. Use was made of the release of ^{51}Cr from labelled target cells as a quantitative measure of cell damage. With this method, the cytotoxic reaction under various experimental conditions has been investigated in greater detail.

MATERIALS AND METHODS

Human liver cells (Chang), obtained from Microbiological Associates, Bethesda, Maryland, U.S.A., were continuously cultivated as spinner cultures. The nutrient medium consisted of Eagle's suspension medium (1959) supplemented with 10 per cent heat-inactivated (30 minutes at 56°) calf serum, 100 units of penicillin and 100 μg of streptomycin per ml. The cells were incubated in siliconized vessels with continuous stirring at 37° in an atmosphere of 95 per cent air and 5 per cent carbon dioxide. Every 4 days the cultures were diluted with 3–4 volumes of fresh medium. After dilution the concentration of living cells was about 3×10^5/ml. At the end of the 4-day period the cultures contained approximately 10^6 viable cells/ml. One day after exchange of medium, more than 90 per cent of the cells were viable as determined by addition of an equal volume of 0·1 per cent trypan blue in buffer. This number decreased to 80–85 per cent towards the end of the culture cycle. For the cytotoxicity experiments the cells were used during the logarithmic growth phase.

Labelling of cells

The method used for [^{51}Cr]chromate labelling of Chang cells *in vitro* was adopted from Sanderson (1964) and Wigzell (1965). Cells from the stock culture were concentrated by centrifugation at 100 ***g***, and about 10^7 living cells in 3 ml of medium were incubated with 100 μc of radioactive sodium chromate for 45 minutes at 37° (sodium [^{51}Cr]chromate, specific activity 100–300 $\mu c/\mu g$ of chromium, Radiochemical Centre, Amersham, England. The cell suspension was chilled in ice water and centrifuged at 100 ***g*** for 5 minutes. The pellet was resuspended and washed four times in ice cold buffer (Hanks's solution, buffered with an equal volume of isotonic Tris, pH 7·4) containing 5 per cent foetal calf serum. Dead cells were identified by trypan blue staining and the cells were counted in a Bürker-chamber with phase contrast at 200× magnification.

Preparation of lymphocytes

Healthy male and female volunteers, 20–60 years old, were used as donors. No selection was made on the basis of blood group. The donors had no history of autoimmune or chronic infectious disease and had not received blood-tranfusion. Fifty to 100 ml of blood was collected from a cubital vein under sterile conditions. The blood was defibrinated by rotation in an Erlenmeyer flask containing glass beads. The lymphocytes were isolated from the defibrinated blood by the method of Coulson and Chalmers (1964). Three volumes of blood were mixed with one volume of 3 per cent gelatin buffered with Hanks–Tris in a siliconized tube. After sedimentation at 37° for 1 hour, the supernatant was pipetted off and centrifuged at 350 ***g*** for 10 minutes. The cells were washed twice in Hanks–Tris buffer. The cells were counted and dead cells identified with trypan blue. Under the present conditions more than 95 per cent of the lymphocytes were unstained.

Phytohaemagglutinin (PHA)

PHA-M (Difco Laboratories, Detroit, Michigan, U.S.A.) was used in all experiments. The content of one vial (100 mg) was dissolved in 5 ml of Hanks–Tris buffer and stored in small portions at $-20°$. The PHA was used shortly after thawing. PHA left over from the experiments was discarded.

Cytotoxicity experiments

The medium used in the cytotoxicity experiments consisted of Parker 199 supplemented with 5 per cent heat-inactivated foetal calf serum (Microbiological Associates, Bethesda, Maryland, U.S.A.) and antibiotics (100 units of penicillin and 100 μg of streptomycin). Glutamine, foetal calf serum and antibiotics were added to fresh Parker medium immediately before use.

The experiments were performed in roller tubes (10×110 mm) under sterile conditions. To each tube were added 10^5 living chromium-labelled Chang cells, $2{\cdot}5 \times 10^6$ living lymphocytes and 0·0075 ml of the stock solution of PHA, in a total volume of 1·5 ml. The cells were suspended by shaking. Each experiment included the following combinations: (a) Chang cells+lymphocytes+PHA; (b) Chang cells+lymphocytes; (c) Chang cells+PHA; and (d) Chang cells only (Table 2). The tubes were incubated at 37° for 24 hours in an atmosphere of 95 per cent air and 5 per cent carbon dioxide. After incubation, the total radioactivity of each tube was determined in a well type scintillation counter (Baird-Atomic, Netherlands). After centrifugation at 350 ***g*** for 5 minutes, 1·0 ml of the cell-free supernatant was withdrawn and its radioactivity determined. The total radioactivity of the supernatant (1·5 ml) was expressed as per cent of the total radioactivity in each tube and was used as a measure of cell damage. All figures of cytotoxicity presented here are the means from two or more independent incubations.

RESULTS

LABELLING OF CELLS

The intensity of labelling of the cells increased with time of incubation with the isotope (Bunting, Kiely and Owen, 1963). However, when the time of incubation exceeded 1 hour the pH of the medium decreased and the cells tended to aggregate and adhere to the glass. Labelling therefore was limited to 45 minutes. Under the conditions described in 'Materials and Methods' the cells were adequately labelled (10 to 20×10^3 counts/min per 10^5 cells). To maintain this level of labelling when the specific radioactivity of the chromate decreased, up to 2 μg of chromium per ml of medium could be added without detectable deleterious effects on the 24-hour survival rate of the cells.

RELEASE OF ISOTOPE

When ^{51}Cr-labelled Chang cells were incubated at 37° in the absence of PHA and lymphocytes, 5–10 per cent of the label was found in the medium within 1 hour. This spontaneous release gradually increased with time of incubation (Fig. 4). It was related to the viability of the cells at the beginning of the experiments. When the samples initially contained less than 20 per cent dead cells, measured by staining with trypan blue, the percentage isotope release after 24 hours incubation was $26{\cdot}3 \pm 1{\cdot}0$ (mean$\pm$S.E. based on twenty-seven experiments) and after 48 hours $41{\cdot}4 \pm 2{\cdot}7$ (five experiments). In cultures

containing initially 25–30 per cent dead cells, more than 40 per cent of the radioactivity was released within 24 hours.

When labelled Chang cells were killed by freezing and thawing and were then incubated at 37°, they released about 70 per cent of their total label to the medium within 1 hour, 80–90 per cent within 24 hours and more than 90 per cent within 48 hours.

Experiments were done to test whether or not dead Chang cells affect the survival of living Chang cells. For this purpose labelled Chang cells were killed by repeated freezing and thawing and were then mixed with living ^{51}Cr-labelled cells in varying proportions. After incubation at 37° for 24 hours the radioactivity of the medium was determined. Fig. 1 shows the per cent release of ^{51}Cr as a function of the proportion of killed cells added

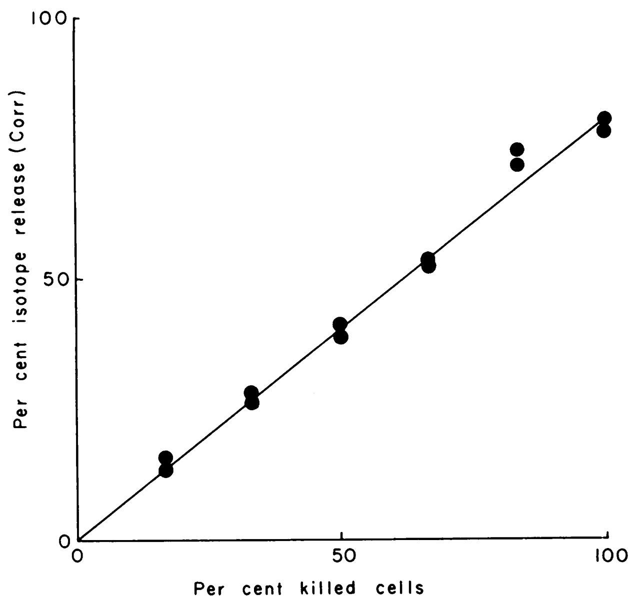

Fig. 1. Correlation between release of isotope and proportion of dead Chang cells. [^{51}Cr]Chang cells, killed by freezing and thawing, mixed with living and labelled Chang cells in different proportions. Total number of cells 10^5/tube. Each point represents a single determination of isotope release after 24 hours incubation. The figures of isotope release are corrected for spontaneous release of radioactivity from living Chang cells.

to the tubes. The isotope recovered from the medium was linearly correlated to the proportion of killed cells.

Further experiments were done to test whether or not re-utilization of isotope occurred. Table 1 shows the results of a typical experiment in which 10^5 ^{51}Cr-labelled Chang cells were distributed to roller tubes and killed by freezing and thawing three times. The killed cells were incubated for 24 hours at 37° in the presence of either 25×10^5 living human lymphocytes, or 10^5 living unlabelled Chang cells. PHA was added to some of the incubations. No living cells were added to the controls. Re-utilization of isotope by living lymphocytes or Chang cells (or adsorption to these) is negligible.

CYTOTOXIC EFFECTS OF LYMPHOCYTES

Complete data from two representative experiments are shown in Table 2. The low spontaneous release of ^{51}Cr in the absence of lymphocytes was only slightly affected by

PHA. In both experiments lymphocytes alone induced some damage of the target cells. In the presence of lymphocytes and PHA the isotope release was about three times higher than the spontaneous release. The cytotoxic effect of lymphocytes can be expressed as the difference between the release in samples containing lymphocytes, and the spontaneous release. This difference has been termed 'corrected cytotoxicity'. In Experiment I of

TABLE 1

THE EFFECT OF LIVING LYMPHOCYTES AND CHANG CELLS ON THE RELEASE OF ^{51}Cr FROM LABELLED CHANG CELLS, KILLED BY FREEZING AND THAWING

Additions to incubation mixture	Isotope recovered in medium*
—	77·8±0·8
PHA	72·8±1·5
Lymphocytes†	74·7±1·2
Lymphocytes†+PHA	69·6±1·0
Chang cells‡	75·7±0·6
Chang cells‡+PHA	73·9±1·7

* Percentage of total isotope present in incubation mixture after 24 hours incubation (mean±S.E., calculated from three independent incubation mixtures.
† 25×10⁵ living lymphocytes.
‡ 10⁵ living, unlabelled cells.

TABLE 2

CYTOTOXIC EFFECTS OF HUMAN LYMPHOCYTES ON CHANG CELLS

Donor	Experiment No.	Chang cells incubated with	Radioactivity: Total counts/min	Radioactivity: Release in per cent of total	Radioactivity: Mean
SH (male)	I	Lymphocytes	11,523 12,893	24·0 24·3	24·2
		Lymphocytes+PHA	11,323 12,185	60·8 62·0	61·4
		No addition	11,909 11,775	20·0 20·8	20·4
		PHA	11,690 11,599	21·6 25·6	23·6
KC (female)	II	Lymphocytes	20,030 19,668	32·8 36·2	34·5
		Lymphocytes+PHA	20,044 21,075	73·2 70·4	71·8
		No addition	20,061 19,713	22·8 23·2	23·0
		PHA	19,399 20,548	23·5 24·7	24·1

Table 2 the corrected cytotoxicity of lymphocytes in the absence of PHA was 3·8 per cent. The corrected cytotoxicity of lymphocytes in the presence of PHA was 41·0 per cent. In thirty-four experiments with lymphocytes from sixteen different donors, the corrected cytotoxicity in the presence of PHA was 29·6±1·5 per cent (mean±S.E.). In the same series, the corrected cytotoxicity of lymphocytes without PHA was 4·3±0·7 per cent.

The cytotoxic effect of lymphocytes from the same donors, tested on different occasions, is shown in Table 3. In the presence of PHA, lymphocytes from donors KH and FA always exerted a high cytotoxicity, with only slight variation from experiment to experiment. Lymphocytes from donors AH and FB seemed to be less cytotoxic but the significance of these results has so far not been established.

TABLE 3

THE CYTOTOXICITY OF LYMPHOCYTES FROM SEVEN DONORS TESTED ON DIFFERENT OCCASIONS ON CHANG CELLS

Donor	Total release of isotope (%)			Corrected cytotoxicity (%)	
	Lymphocytes +PHA	Lymphocytes	No addition	Lymphocytes +PHA	Lymphocytes
KH (female)	68·0	32·0	30·8	37·2	1·2
	57·7	26·1	24·4	33·3	1·7
	56·5	25·9	23·8	32·7	2·1
	53·8	26·0	21·5	32·3	4·5
	59·7	31·0	28·0	31·7	3·0
MG (male)	70·7	36·0	25·0	45·7	11·0
	49·1	25·8	19·2	29·9	6·6
	49·0	37·1	24·8	24·2	12·3
	48·5	29·9	25·0	23·5	4·9
	58·7	39·7	37·0	21·7	2·7
GH (male)	74·7	42·1	35·4	39·3	6·7
	60·2	33·8	24·7	35·5	9·1
	47·2	25·1	22·0	25·2	3·1
	41·5	26·8	21·8	19·7	5·0
	43·9	28·7	24·4	19·5	4·3
FA (male)	61·3	29·4	24·0	37·3	5·4
	57·7	21·7	24·6	33·1	−2·9*
	52·5	29·2	22·3	30·2	6·9
AJ (male)	68·4	35·8	31·3	37·1	4·5
	55·7	33·0	24·4	31·3	8·6
	47·1	32·9	30·3	16·8	1·6
AH (female)	44·9	32·8	27·2	17·7	5·6
	38·9	30·9	27·9	11·0	3·0
FB (male)	48·6	33·4	25·8	22·8	7·6
	45·6	32·6	23·1	22·5	9·5

* Cytotoxicity of lymphocytes lower than spontaneous release.

INFLUENCE OF CONTAMINATING BLOOD CELLS ON THE CYTOTOXIC REACTION OF LYMPHOCYTES

The lymphocytes prepared by sedimentation in gelatin were always contaminated with red cells. It has been found that red cells, agglutinated to allogeneic target cells by means of PHA, were not cytotoxic even when the ratio of red cells to target cells was 1000:1 (Holm and Perlmann, 1965). The influence of contaminating red cells on the cytotoxic reactivity of lymphocytes was studied by comparing the cytotoxicity of lymphocytes before and after lysis of red cells. In a typical experiment lymphocytes, prepared and centrifuged as described, were treated for 30 seconds with 2 ml of 0·05 M sodium chloride. The cell suspension was then diluted with 5 volumes of isotonic buffer containing 5 per cent serum. The cells were washed three times and counted. The untreated control contained white cells (90 per cent lymphocytes) and red cells in the ratio of 1 to 15. After treatment, the small lymphocytes were contaminated with less than 5 per cent red cell ghosts and some granulocytes. More than 90 per cent of the lymphocytes were alive by

trypan blue exclusion. The hypotonic treatment did not reduce their capability to synthesize DNA and to transform into blastoid cells after addition of PHA (unpublished observation). The maximal cytotoxicity of lymphocytes exposed to hypotonic saline was similar to that of non-treated cells (Fig. 2). At low concentration of PHA, contaminating

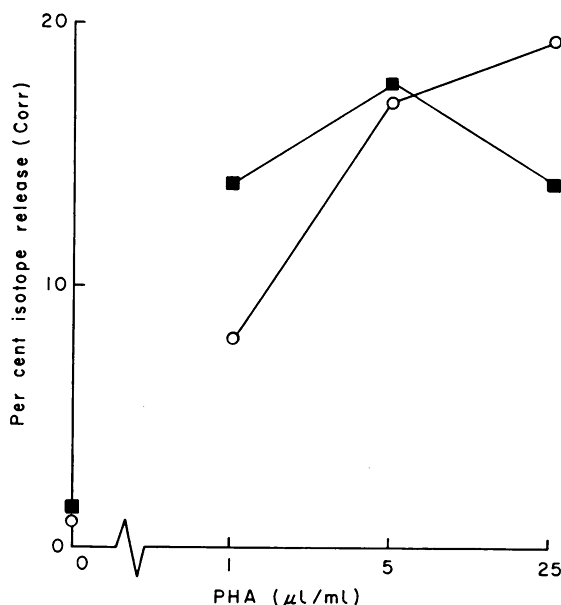

FIG. 2. Cytotoxic effect of lymphocytes before and after lysis of contaminating red cells. ○, Control, no pre-treatment of lymphocytes; ■, lysis of red cells by hypotonic treatment before experiment.

red cells reduced the cytotoxicity of lymphocytes, and moreover, red cells delayed the inhibition of cytotoxicity regularly observed at high concentration of PHA.

The influence of contaminating granulocytes on the cytotoxic reaction can be seen from the typical data in Table 4. In this experiment, the white cells, prepared from the

TABLE 4

THE EFFECT OF GRANULOCYTES ON THE CYTOTOXICITY OF LYMPHOCYTES ON CHANG CELLS

Chang cells incubated with	Corrected cytotoxicity (%)	
	PHA added	No PHA
Lymphocytes	21·7	2·7
Granulocytes	−0·8*	3·0
Lymphocytes + granulocytes	21·0	11·7

* Cytotoxicity lower than spontaneous release.

gelatin supernatant, contained 86 per cent small lymphocytes. Granulocytes were obtained from the buffy coat of the same gelatin sedimentation (85 per cent granulocytes). Labelled Chang cells (10^5) were incubated with 25×10^5 lymphocytes from the lymphocyte-rich preparation and/or 25×10^5 granulocytes from the granulocyte-rich preparation. Granulocytes alone did not cause cell damage. The cytotoxicity of lymphocytes in the presence of PHA was not altered by the admixture of granulocytes. In the absence of PHA the

mixture of lymphocytes and granulocytes caused a higher release of radioactivity than either of these preparations alone.

EFFECT OF DIFFERENT MEDIA AND SERA ON THE CYTOTOXIC REACTION

The cytotoxicity of lymphocytes from one donor was tested in five different combinations of medium and serum (Table 5) and determined after 24 hours incubation. Some cell damage was observed in all media in the absence of PHA. This damage was more pronounced when Eagle's medium was used. The lowest 'spontaneous' cytotoxicity of lymphocytes was seen in Parker's medium containing 5 per cent homologous human serum

TABLE 5
EFFECT OF DIFFERENT MEDIA ON THE CYTOTOXICITY OF LYMPHOCYTES ON CHANG CELLS

Medium	Serum (5%)	Total isotope release (%)		
		Lymphocytes +PHA	Lymphocytes	No addition
Parker 199	Human	73·8	45·3	34·7
	Foetal calf	74·3	45·3	32·2
	Adult calf	72·8	52·9	33·8
Eagle	Human	69·2	51·0	30·8
	Foetal calf	72·5	57·2	28·3

or foetal calf serum. Adult calf serum caused some mixed aggregation which could explain the high cell damage in these tubes. No aggregation was observed when other sera were used. The level of the cytotoxicity of lymphocytes in the presence of PHA was usually not influenced by the choice of medium and serum. Other experiments showed that an increase of the concentration of foetal calf serum from 5 to 15 per cent did not affect the cytotoxicity of the lymphocytes. The results indicate that Parker's medium 199 containing 5 per cent foetal calf serum or homologous human serum provided the most suitable conditions for the cytotoxicity reaction.

EFFECT OF THE LYMPHOCYTE/TARGET CELL RATIO ON THE CYTOTOXIC REACTION

Labelled Chang cells (10^5) were exposed to 10^4 to $12{\cdot}5 \times 10^6$ lymphocytes for 24 hours. The results from five independent experiments with five different lymphocyte donors are presented in Fig. 3. The corrected cytotoxicity of the lymphocytes in the presence of PHA is given on the ordinate and the ratio of lymphocytes to Chang cells on the abscissa (log scale). Where lymphocytes were in excess, the cytotoxicity increased rapidly with increasing ratios. A weak cell damage was also seen at ratios lower than 1:1. The ratio response curve was sigmoid.

THE KINETICS OF THE CYTOTOXIC REACTION: ^{51}Cr AND THYMIDINE-^{14}C RELEASE FROM DOUBLE-LABELLED TARGET CELLS

Chang cells growing in suspensions were labelled both with [^{14}C]thymidine (Holm, 1966) and [^{51}Cr]chromate. Washed cells (10^5) were incubated with 25×10^5 lymphocytes with and without PHA. Controls with only Chang cells were included. Two tubes of each mixture were harvested simultaneously after incubation at 37° for various periods of time.

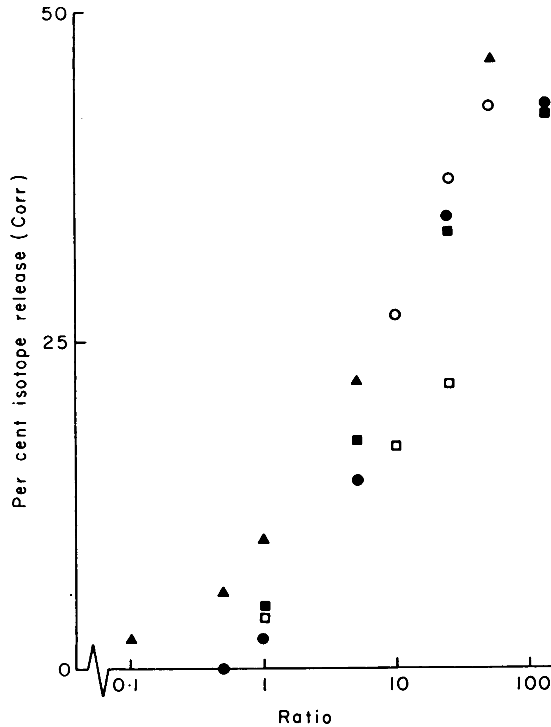

FIG. 3. Cytotoxic effect of lymphocytes at different lymphocyte–target cell ratios. Five independent experiments with lymphocytes from five different donors. Each symbol represents the mean of two independent incubations.

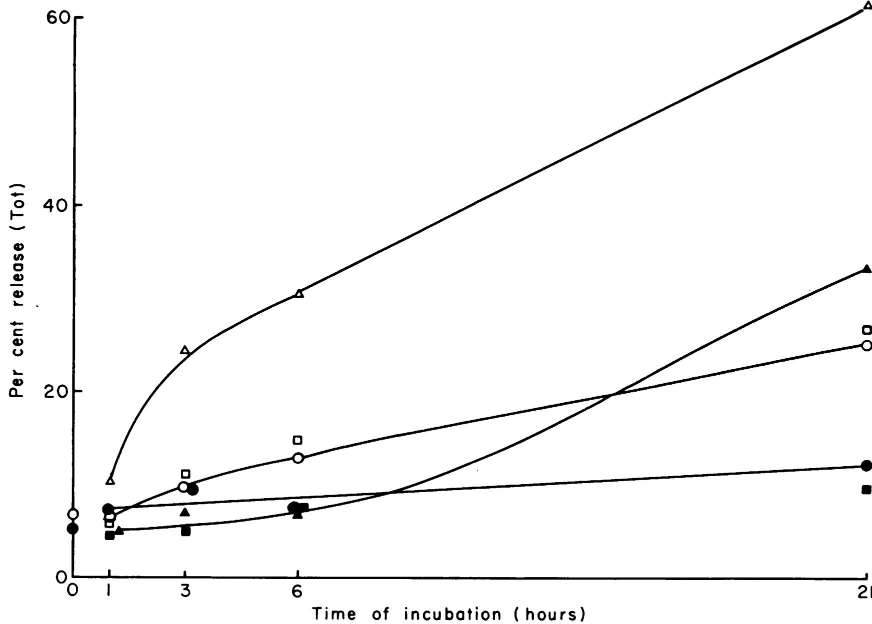

FIG. 4. Cytotoxicity of lymphocytes as a function of time of incubation. Comparison between release of ^{51}Cr and [^{14}C]thymidine from Chang cells labelled with both isotopes. ○, ^{51}Cr, control; □, ^{51}Cr, lymphocytes added; △, ^{51}Cr, lymphocytes+PHA added; ●, [^{14}C]thymidine, control; ■, [^{14}C]-thymidine, lymphocytes added; ▲, [^{14}C]thymidine, lymphocytes+PHA added.

The gamma irradiation of ^{51}Cr was determined as usual. For determination of the release of [^{14}C]thymidine from damaged cells the sediments were treated with tryspin and centrifuged. After extraction of DNA with TCA, the beta irradiation in the supernatant and cell residue was measured in a Geiger–Müller counter as described earlier (Holm, 1966).

The results are presented in Fig. 4. The release of ^{51}Cr was always higher than the corresponding release of ^{14}C. The spontaneous release of ^{51}Cr from Chang cells in the absence of lymphocytes increased slowly from 7 per cent after 1 hour to 25 per cent after 21 hours incubation. Lymphocytes without PHA caused only a small increase of the release of ^{51}Cr above the controls. However, when PHA was present, lymphocytes provoked loss of 25 per cent of the cellular ^{51}Cr into the medium during the first 3 hours of incubation. There was no release of [^{14}C]thymidine at 1, 3 or 6 hours of incubation. After 21 hours of incubation the percentage release of thymidine caused by lymphocytes in the presence of PHA was about 2·5 times higher than that of the controls. However, it was only two-thirds of the percentage of ^{51}Cr-release at the end of the experiment.

DISCUSSION

The release of ^{51}Cr from labelled lymphocytes or tumour cells has been shown to be a convenient and accurate measure of cell damage induced by antibody (Goodman, 1961; Sanderson, 1964; Wigzell, 1965). This method is also suitable for the study of the cytotoxic effect of lymphocytes on cells in tissue culture. The isotope is bound to cellular structures in an unknown manner. There was no release of isotope from Chang cells when incubated for 3 hours at 4°. Moreover, only 6 per cent of the total label was found in the medium when Chang cells were incubated for 24 hours at 20° (unpublished observations). When incubated at 37° the spontaneous release was about 25 per cent. Incubation at this temperature for 48 hours led to a release of 40–50 per cent. Probably this radioactivity originated from cells that were dead at the beginning of the experiment or had died during the course of incubation. It can not be excluded, however, that some radioactivity was released from intact cells.

The nature of the spontaneous release of ^{51}Cr from the Chang cells in the absence of lymphocytes during the 24 hours of incubation at 37° is not completely understood. When cell damage is assessed by determination of the release of [^{14}C]thymidine, the spontaneous release at 24 hours of incubation is only slightly higher than that found at the beginning of the experiment (Fig. 4). This label, which is localized to the cell nuclei, is only released from damaged cells after treatment with trypsin (Klein and Perlmann, 1963). Hence, it is likely that ^{51}Cr-release in the absence of lymphocytes represents a less profound cell damage which is not revealed when other criteria for 'cell death' are applied. It should be stressed that the ^{51}Cr of the medium represents the accumulated release during the course of incubation. It can not be compared with figures for cell death obtained with vital staining techniques, which are influenced by cell division and disappearance of dead cells during the period of incubation.

Re-utilization of isotope would tend to decrease the sensitivity of the present method. The data presented here indicate no re-utilization during 24 hours of incubation. This confirms and extends the observation by Bunting *et al.* (1963), who could not demonstrate re-utilization in short term experiments.

Granulocytes or erythrocytes did not influence the cytotoxicity of lymphocytes at the

standard concentration of PHA. We have found that the cytotoxic effect of lymphocytes is a function of the concentration of PHA (to be published). As seen from Fig. 2, the presence of erythrocytes led to a shift of the dose–response curve to the right. This may reflect a consumption of PHA by red cells, or some other interference with the lymphocyte–Chang cell aggregation.

When labelled Chang cells, adapted to growth in suspension, were allowed to settle on glass before lymphocytes and PHA were added, cell damage was weaker than observed when cells and PHA were mixed in suspension (unpublished observations). It is reasonable to assume that the total surface of cells in suspension is accessible to lymphocytes, whereas monolayer cells expose only a part of their surface. It could also be argued that lymphocytes aggregated to the surface of Chang cells by PHA may prevent the attachment of the latter to the glass. This would create unfavourable conditions for the cells and thus lead to their damage. However, no cell damage was observed when the lymphocytes were replaced by other non-lymphoid cell types (Holm and Perlmann, 1965; data to be published). Moreover, when the lymphocyte–target cell ratio was less than 1:1, cytotoxic effects were also demonstrated. At such small ratios, the lymphocytes could scarcely have prevented the attachment of Chang cells to the glass.

The cell damaging effect of lymphocytes on tissue culture cells has been assumed to be a slow process which needs 24–48 hours to become manifest (Rosenau and Moon, 1961; Wilson, 1965). When determined by measuring ^{51}Cr-release from labelled target cells, it was found to be rapid and a significant release from Chang cells was observed after 1 hour of incubation (Fig. 5). The rapid initial increase of radioactivity in the medium may represent the early escape from the cells of constituents, which were released when the cell membranes were only slightly damaged. The slow release of ^{51}Cr during the later phase may then represent a more pronounced injury. It is also possible that a fragile population of Chang cells was rapidly damaged, whereas the rest was more resistant to the action of lymphocytes. However, since no early cell damage was detected when the release of [^{14}C]thymidine was measured, the first explanation appears more likely.

Some information on the cytotoxic efficiency of lymphocytes was obtained from experiments in which the lymphocyte–target cell ratios were varied. The relationship between the percentage of isotope release and the logarithm of the ratio followed a sigmoid curve (Fig. 4). Complete lysis of Chang cells at 24 hours corresponded to about 60 per cent *corrected* cytotoxicity (= about 80 per cent uncorrected release, see Table 1). The corrected cytotoxicity of Fig. 4 could be recalculated on the assumption that 60 per cent corrected isotope release was equal to complete cell lysis (= 100 per cent damaged cells). This gave rise to an S-shaped dose response curve which could easily be converted to a straight line by probit transformation. The dose for 50 per cent lysis, obtained from this curve, corresponded to lymphocyte target cell ratios between 5:1 and 25:1. Assuming that close contact between lymphocytes and Chang cells was the most important factor for damage of the latter (Holm *et al.*, 1964; Möller, 1965) a considerable proportion or lymphocytes must have been active and able to kill Chang cells within 24 hours. The cytotoxic effect might be exerted by initially active and aggregated lymphocytes or by lymphocytes, which became active during the experiment by contact with the target cells or by transfer of information from a smaller proportion of active lymphocytes. However, from the present data it can not be excluded that contact between target cells and certain lymphocytes causes release of toxic agents. Such a mechanism would also result in a relatively high cell-damaging efficiency of the lymphocytes at low ratios.

It has been found that PHA-mediated aggregation of lymphocytes from non-sensitized animals to syngeneic target cells in tissue culture under similar experimental conditions as used here, did not lead to damage of the latter. In contrast, cell damage was observed in allogeneic and xenogeneic systems (Holm and Perlmann, 1965; Möller, 1965). The cytotoxic effects noted in this paper may reflect the reaction between lymphocytes and foreign antigenic structures on the Chang cells. The intensity of the reaction might then be related to the histoincompatibility between the cells. The differences of cytotoxicity observed with lymphocytes from different donors (Table 3) would in fact speak in favour of this hypothesis. However, more experiments are needed to settle this important question.

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