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CYTOTOXICITY OF LYMPHOCYTES FROM HEALTHY SUBJECTS AND FROM MELANOMA PATIENTS AGAINST CULTURED MELANOMA CELLS

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SUMMARY

The *in vitro* cytotoxicity of lymphocytes from forty-seven melanoma patients and thirteen healthy subjects for cultured melanoma cells was studied using a ^{51}Cr release assay. Two different melanoma cell lines were used as target cells: one cultured in suspension (SK Mel_1) and one tissue culture line growing as a monolayer (NK I_1). The lymphocytes from most healthy subjects were found to be cytotoxic for these cultured cells, with individual variations. These repeatable cytotoxic reactions could not be explained on the grounds of previous isoimmunization. The lymphocytes from melanoma patients were also cytotoxic for the melanoma cell lines, but the highest degree of cytotoxicity was found in patients with primitive and localized tumours, and not in patients with metastases.

INTRODUCTION

In human malignant melanoma, as in many other human tumours, investigation of cell-mediated immune reactions *in vitro*, using the microcytotoxicity assay (MA) (Takasugi & Klein, 1970) have defined cell-mediated cytotoxic reactions in most melanoma patients, against syngeneic and allogeneic melanoma cells (De Vries, Rümke & Bernheim, 1972; Fossatti *et al.*, 1971; Byrne *et al.*, 1973) with serum blocking factors during the invasive phase (Hellström *et al.*, 1973; Heppner *et al.*, 1973), and unblocking activity following regression (Hellström *et al.*, 1971).

The release of ^{51}Cr from labelled target cells can quantificate membrane damage induced by immune lymphocytes (Brunner *et al.*, 1968), and represents a well documented technique for the detection of cell-mediated immune reactions against allogeneic cells (Brunner *et al.*, 1970), as well as syngeneic tumours (Leclerc, Gomard & Levy, 1972). In experimental virus-induced tumours, evidence exists that this ^{51}Cr release test (CRT) does not reveal exactly the same cell-mediated immune reactions *in vitro* as the microcytotoxicity assay

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(MA). In such models, the CRT detects only a T cell-mediated cytotoxicity (Leclerc *et al.*, 1973), which is not blocked by soluble tumour antigens (Plata & Levy, 1974), nor by serum from tumour-bearing animals, whereas the cytostatic effect in MA is mediated by both T and non-T cells (Plata *et al.*, 1974), and is blocked by soluble antigens (Plata & Levy, 1974) or serum from tumour-bearing mice (Hellström & Hellström, 1969; Skurzak *et al.*, 1972; Plata *et al.*, 1974). This suggests that CRT and MA may involve different mechanisms of cellular cytotoxicity and/or different subpopulations of effector cells.

In the present work, we have used a ^{51}Cr release test to evaluate the *in vitro* cell-mediated cytotoxicity in patients with malignant melanoma, against two different types of tissue culture melanoma cell lines. In our experimental conditions, lymphocytes from most healthy subjects were found to be toxic for these cultured tumour cells. The lymphocytes from melanoma patients were also cytotoxic, but the highest level of cytotoxicity was found among patients with primitive and localized tumours.

MATERIALS AND METHODS

Target cells

Cultured, rather than fresh tumour cells were chosen, because of the poor viability of melanoma cells dissociated from biopsy specimens in preliminary experiments. Two types of melanoma cell lines in culture were used. The line SK Mel_1 (Oettgen *et al.*, 1968), kindly provided by E. Beth, was grown in suspension in a mixture of RPMI 1640 and Eagle's minimal essential medium (MEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), and tested between passages 51 and 90. The HL-A type determined by J. Dausset, was HL-A 10, 11, 7 and W5. A weak membrane immunofluorescence reaction with allogeneic melanoma sera, provided by J. F. Doré, was observed. The NK I_1 cell line (ME2C) was kindly provided by J. De Vries (Antoni Van Leeuwenhoekhuis, Amsterdam), grown as a monolayer in Eagle's MEM supplemented with 10% FCS, L-glutamine and antibiotics, and assayed between passage 180 and 200. The HL-A type, as determined in Amsterdam on both cultured cells and patient's lymphocytes was HL-A 2, 13, W17, 4a and 4b. This melanoma cell line gave positive reactions with leucocytes from melanoma patients, in microcytotoxicity assays (De Vries *et al.*, 1972; personal communication).

Control experiments were made using PHA lymphoblasts as target cells: lymphocytes isolated from the peripheral blood of healthy donors by Ficoll–Triosil gradient centrifugation, were cultured at a concentration of 10^6/ml for 3 days in the presence of a 1 : 100 dilution of PHA-M (Difco) and washed before labelling with chromium. In blocking experiments, fibroblasts derived from the normal skin of a melanoma patient, and a lymphoblastoid cell line LHN12, established from a normal subject by C. Rosenfeld, were also used.

Lymphocyte donors

Lymphocytes were obtained from forty-seven melanoma subjects with histologically confirmed tumours and from two cases of Hutchinson's freckles. We classified the melanoma patients into two groups: (a) primitive localized tumours with no detectable lymph node involvement (twenty-three cases); most patients were bled after the biopsy and before surgical removal of the tumour; (b) metastatic and recurrent tumours including tumours with regional lymph node involvement, diffuse metastasis and local recurrences (twenty-

four cases). A number of the patients (twenty-five) were tested several times during the course of the disease, in the absence of chemotherapy for the patients having localized tumours. The absence of previous blood transfusion was controlled.

Normal lymphocytes were obtained from thirteen healthy donors. Previous history of transfusion or multiparity was found *a posteriori* in three of them. Anti-HL-A immune lymphocytes were obtained from a healthy volunteer (D.U.P.) (immunized in the laboratory of J. Dausset), bled at 0, 15 and 21 days following a single subcutaneous injection of 20×10^6 allogeneic lymphocytes from an HL-A incompatible brother.

Lymphocyte suspensions

They were obtained by a sterile procedure from defibrinated peripheral blood following Ficoll–Triosil gradient separation (Boyüm, 1968). The cells were washed and resuspended in MEM supplemented with 20% FCS. The final cell suspensions contained more than 90% mononucleated cells and very few erythrocytes and are referred to as 'lymphocyte' suspensions. From one healthy donor (M.O.L.), a large number of mononuclear cells was obtained with an experimental blood cell separator IBM 2990. Following preliminary experiments which failed to show any significant difference between fresh and frozen lymphocytes in CRT, most lymphocyte suspensions used were frozen in the presence of DMSO, at a cooling rate of 1°C/min, with a Linde BF4-1 freezing apparatus, and stored in liquid nitrogen (Flynn, Troup, Walford, 1966). Before test, the cells were thawed using a stepwise dilution procedure, washed and resuspended in MEM with 20% FCS. The viability of the cell suspensions was always over 80%.

Cr release test

The technique was derived from that of Brunner *et al.* (1970). Cells in suspensions from SK Mel_1, or cells obtained by gentle trypsinization from NK I_1 monolayers were washed three times and labelled with $^{51}CrO_4Na$ (Centre de l'Energie Atomique, Gif-sur-Yvette). Fifty or 100 μCi were added to 1 or 5×10^6 cells in 1 ml of serum-free MEM, and incubated for 45 min at room temperature with occasional shaking. Following two washings, the labelled cells were resuspended in MEM supplemented with 20% FCS at a concentration of 10^5 cells per millilitre. Each reaction tube received 0·1 ml of the labelled cell suspension (10^4 cells) and 1 ml of the lymphocyte suspension (10^6 cells). This standard lymphocyte/target cell ratio (L/T) of 100:1 was chosen following preliminary experiments. Each experimental series included: (a) target cells mixed with lymphocytes from melanoma patients; (b) target cells with lymphocytes from healthy donors; (c) target cells alone, to determine the spontaneous ^{51}Cr release and (d) target cells lysed in distilled water, to evaluate the maximum ^{51}Cr release.

Triplicate tubes were incubated at 37°C in humidified air with 5% CO_2 and harvested at different times, e.g. 1, 4, 8, 18 and 20 or 22 hr. In many experiments where the amount of lymphocytes was limited, the longer incubation time (20–22 hr) was chosen. Following centrifugation at 200 *g* for 10 min, 0·5 ml of supernatant from each test tube (A) was transferred into another tube (B), and the radioactivity of tubes A and B counted in an automatic well-type gamma counter (Packard Instrument Company, Downer's Grave, Illinois). The percentage of Cr release was determined as follows.

$$\text{Percentage Cr release} = \frac{\text{ct/min in tube B} \times 2{\cdot}2}{\text{ct/min in tube A} + \text{ct/min in tube B}} \times 100.$$

The results given are the mean percentage from triplicate reactions.

Preliminary assays were made to define the parameters of our experimental conditions The ^{51}Cr uptake was higher for NK I_1 than for SK Mel_1 and varied from 200 to 800 ct/min per 10^3 cells. The maximum ^{51}Cr release following osmotic lysis was higher from SK Mel_1 than from NK I_1 and represented 80–90% of the total ^{51}Cr uptake. The rate of the spontaneous Cr release from both cell lines showed some variation from one experiment to another, as illustrated in Fig. 1, for SK Mel_1 cell line. The figure of spontaneous ^{51}Cr release was therefore taken into account in each experiment (see below). A few experiments where the spontaneous ^{51}Cr release was greater than 50% at 24 hr were discarded.

For blocking experiments, the cold cells (NK I_1, fibroblasts or LHN_{12}) were added to the standard mixture of 10^6 lymphocytes and 10^4 labelled target cells. Ratios of cold *vs* labelled cells ranging from 7 : 1 to 30 : 1 were used.

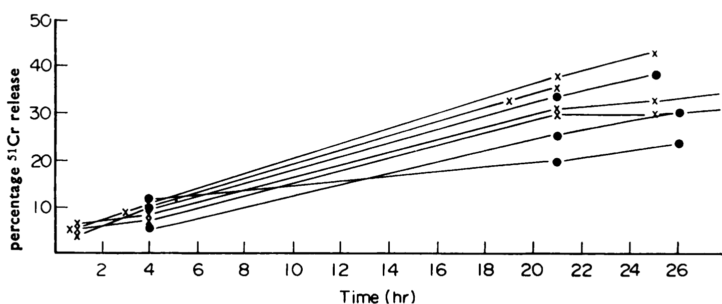

FIG. 1. Variations in the spontaneous ^{51}Cr release from cultured SK Mel_1 cell line in seven separate experiments.

RESULTS

Cytotoxicity of immune allogeneic lymphocytes

In order to define the range of cytotoxicity to be expected from immune lymphocytes under our experimental conditions, the lymphocytes from a healthy volunteer (D.U.P.) immunized with allogeneic, HL-A-incompatible leucocytes, were used as effectors against NK I_1 target cells in our CRT. Fig. 2 shows that the lymphocytes D.U.P., taken at day 15 following immunization, increase by 21% the ^{51}Cr release at 21 hr as compared to lymphocytes from the same donor before immunization, or to the lymphocytes of a normal donor (M.O.L.) previously selected for their low level of spontaneous cytotoxicity. When these experiments, using frozen lymphocytes, were repeated, the same order of magnitude of increase of Cr release was found.

Cytotoxicity of lymphocytes from healthy individuals in CRT

The effect of lymphocyte suspensions from healthy subjects was determined in CRT using SK Mel_1 as target cells. The results of twenty-eight tests performed with frozen lymphocytes suspensions from thirteen healthy donors are given in Fig. 3. As a rule, the ^{51}Cr release from SK Mel_1 over a 20 hr incubation period, is significantly increased in the presence of normal lymphocytes (mean increase = $6{\cdot}6 \pm 7{\cdot}4$). This increase can be significant, reaching 35% in one case, but remaining below 10% in the majority of cases. The

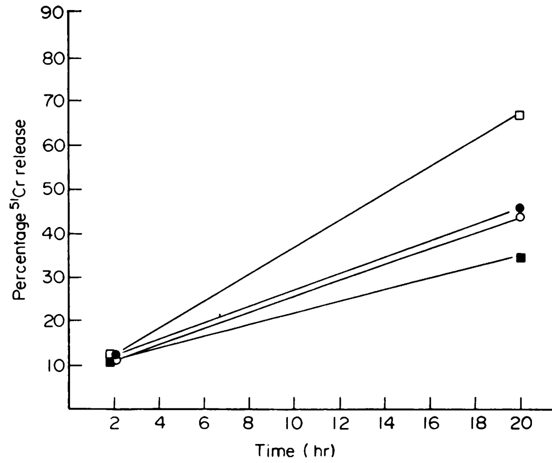

FIG. 2. ^{51}Cr release from NK I_1 cells induced in CRT by isoimmune lymphocytes (D.U.P.). (●) Lymphocytes D.U.P. before immunization. (□) Lymphocytes D.U.P. after immunization. (○) Lymphocytes from a normal donor (M.O.L.). (■) No lymphocytes (spontaneous release).

same frozen lymphocyte suspensions, repeatedly tested, gave similar results. When lymphocytes from the same subjects bled at different times were compared, approximately the same level of cytotoxicity was found, except in one instance, where the lymphocytes became more toxic at 2-months interval. Comparing the effects of the same lymphocyte suspensions before and after freezing, indicated that frozen-stored lymphocyte preparations were regularly less toxic than fresh ones. A retrospective search for iso-immunization revealed previous transfusions and multiparity in three donors, shown in Fig. 3, failed to provide an explanation for the toxic effect of lymphocytes from the other subjects.

Using NK I_1 as target cells, the increase in ^{51}Cr release induced by a reference lymphocyte suspension (M.O.L.) was repeatedly found more important than that observed with SK Mel_1 cells, using the same lymphocytes, under the same experimental conditions. This may indicate a greater sensitivity of NK I_1 cells to lymphocytotoxicity.

Since the lymphocytes from most healthy subjects tested were toxic to a variable extent for both cell lines, no correct control figure of a 'normal cytotoxic effect' can be defined,

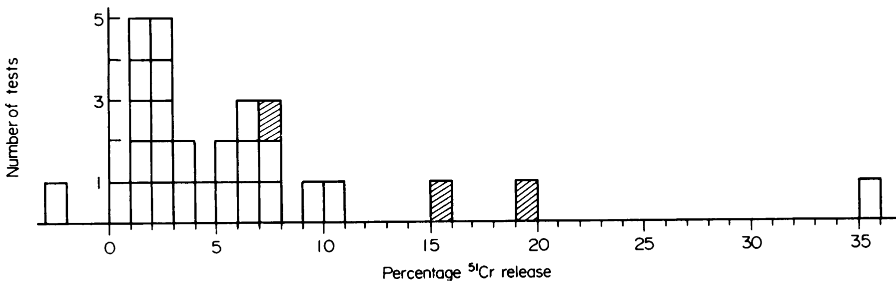

FIG. 3. Cytotoxic effect of lymphocytes from healthy subjects for ^{51}Cr-labelled SK Mel_1 cells. Results are expressed as the increase in ^{51}Cr release. The hatched squares indicate the three donors with a previous history of isoimmunization.

within the limits of the small number of healthy subjects tested. However, since more than 90% of tests performed with normal lymphocytes at a L/T ratio of 100 : 1 yielded an increase in ^{51}Cr release of less than 10% for SK Mel_1 and less than 15% for NK I_1 cell line, these values were taken as the arbitrary basis for a comparison with the lymphocytes from melanoma patients.

Cytotoxocity of lymphocytes from melanoma patients

The cytotoxic effect of lymphocytes from melanoma patients against SK Mel_1 cell line in the CRT at 20 hr is illustrated in Fig. 4. Thirty patients were tested, including eleven

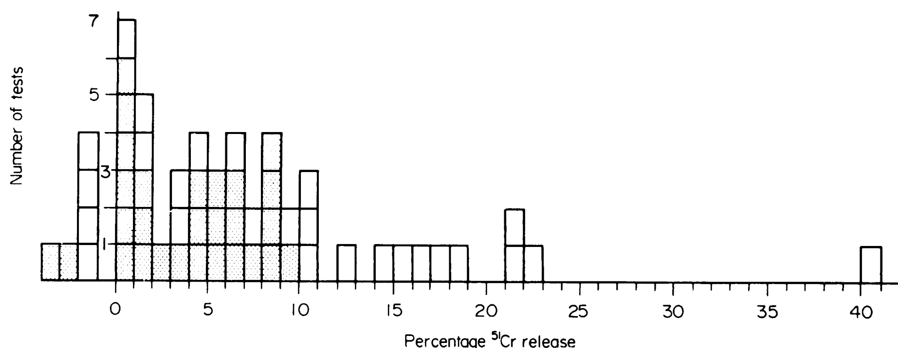

FIG. 4. Cytotoxic effect of lymphocytes from melanoma patients for ^{51}Cr-labelled SK Mel_1 cells. Open squares, patients with primitive and localized tumours, stippled squares, patients with recurrent tumours or metastases.

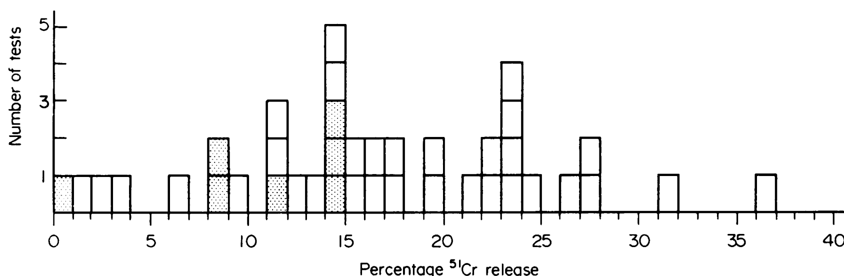

FIG. 5. Cytotoxic effect of lymphocytes from melanoma patients for ^{51}Cr-labelled NK I_1 cells. Open squares, patients with primitive and localized tumours. Stippled squares, patients with recurrent tumours or metastases.

patients with primitive and localized tumours without chemotherapy and nineteen patients with metastatic or recurrent tumours. Fourteen patients were tested at time intervals, and the results obtained with fifty-three lymphocyte suspensions are shown in Fig. 4. One can see that, on the whole, the increase in ^{51}Cr release over the spontaneous release from SK Mel_1, in the presence of lymphocytes from melanoma patients (mean increase $7{\cdot}8 \pm 7{\cdot}7$), is not significantly different from that observed using lymphocytes from healthy subjects (mean increase $6{\cdot}6 \pm 7{\cdot}4$) (see Fig. 3). However, it is noteworthy that the highest figures of ^{51}Cr release (greater than 10%) concern lymphocytes from seven patients with primitive

and localized tumours. The figures for two patients with Hutchinson's freckles were 6 and 11% of ^{51}Cr release, respectively.

Comparable results were obtained using NK I_1 cells as targets. In this case, nineteen patients (six with metastatic, and thirteen with primitive tumours without chemotherapy) were tested, eleven of them at time intervals, and results obtained with thirty-nine lymphocyte suspensions are shown in Fig. 5. Most lymphocyte suspensions increase the spontaneous ^{51}Cr release from NK I_1, (mean increase = $16{\cdot}6 \pm 8{\cdot}3$). This increase is greater than 15%, for twenty one lymphocyte-suspensions, which come from nine patients, all with primitive and localized tumours.

Comparison between lymphocyte cytotoxicity of melanoma patients and normal subjects

In the results reported above, the choice of the spontaneous ^{51}Cr release from target cells as the baseline for evaluating the cytotoxic effect of added lymphocytes is not satisfactory, since the culture conditions are not comparable upon addition of 100 times more

TABLE 1. Distribution of CRT 'positive' and CRT 'negative' melanoma patients according to the stage of the disease

Target cells in CRT	Number positive*/number tested		
	All patients	Primitive tumours	Metastasis
SK Mel-$_1$	4/30	4/12	0/18
NK I_1	9/19	9/13	0/6
Total	13/47	13/23	0/24

* Criteria for positivity were established by comparison with random lymphocytes in the case of SK Mel_1 and a 'standard' lymphocyte in the case of NK I_1 (see text).

cells. In fact, *in vitro* cell-mediated cytotoxicity assays usually compare the effect of lymphocytes from cancer patients and non-cancerous subjects within the same experiment. Attempts were made to achieve such a comparison, although the unpredictable toxicity of control lymphocytes from normal donors selected at random on a given day renders this approach somewhat arbitrary.

The results given in Table 1 take into account the difference between the ^{51}Cr release induced by lymphocytes from melanoma patients and from the healthy subject tested in the same experiment on the same day. Using SK Mel_1 as target, we have arbitrarily considered as positive all tests where the ^{51}Cr release induced by patients' lymphocytes was more than 10% greater than that induced by normal control lymphocytes. Only four out of thirty-patients fulfil this arbitrary criteria of positivity in at least one test, but all belong to the group of patients with localized tumours.

Using NK I_1 as target cells, another approach was made (Table 1). In order to circumvent the problem of individual variations of the toxicity of normal lymphocytes chosen at random as controls, we have taken for the whole series of tests the same 'standard' lymphocyte suspension from a single individual (M.O.L.), stored in frozen aliquots. Considering a ^{51}Cr release equal to or more than 10% above the level given by control lymphocytes on the

same day as the criteria for positivity, again all nine positive patients fall into the group with localized tumours, but, in this series, metastatic cases are under-represented. Only two lymphocyte suspensions were tested against both cell lines, but were among the negative tests.

Cytotoxocity of lymphocytes from patients taken at time intervals

Lymphocytes could be obtained at time intervals from twenty-five patients. Eleven (one with metastasis, and ten with primitive tumours) were tested using NK I_1 target cells, and fourteen (seven primitive cases and seven with metastasis or recurrence) were tested

TABLE 2. Evolution of cytotoxic reactions of twelve melanoma patients showing at least one 'positive' CRT

		Results of CRT*											
Patient	Target cell in CRT	Before surgery	Months after surgery										
			0	1	2	3	4	5	6	7	8	9	0
P.A.V.	NK I_1	+						+					
G.A.L.	NK I_1	−	−		+		+						
M.I.R.	NK I_1		−	−		+		+					
F.O.N.	NK I_1	−	+	+	+							R†	
L.E.U.	NK I_1	+	−	−	+			+					
J.O.U.	NK I_1	+	−		+								
G.A.M.	NK I_1	−						+					
G.O.D.	NK I_1			+				−					
S.T.R.	SK Mel_1	+	+‡										
L.E.M.	SK Mel_1		−		+					R†			
B.E.R.	SK Mel_1	+	+	−									
B.U.S.	SK Mel_1	−	+	±									

* Positive if increase of ^{51}Cr release is more than 10% over that of normal lymphocytes taken as a control in the same experiment (see text).

† R = recurrence.

‡ Death.

against SK Mel_1 target cells. CRT were performed two to five times in each patient over a period varying from 1 week to 10 months after the first test. Variations in the reactivity of lymphocytes were observed, and are schematically represented in Table 2, which deals only with the twelve patients in whom a 'positive' cytotoxic reaction was observed at least once, according to the criteria defined in the above paragraph (10% ^{51}Cr release more than that of control lymphocytes). These variations may be in part attributed to variations in the cytotoxic level of lymphocytes taken as controls, and to technical conditions, although several tests repeated with the same leucocyte preparations yielded identical results.

No obvious correlation between 'positive' CRT and clinical status or prognosis of patients with primitive tumours was found, and the 'negativity' of CRT in four cases during the 1–2 months following surgical removal of tumour could not be explained by chemotherapy or radiotherapy.

Specificity assays

In an attempt to assess if the cell-mediated cytotoxicity observed in CRT toward melanoma cell lines was non-specific or related to melanoma-associated properties (antigens?), two preliminary approaches were made.

Trials to inhibit cell-mediated cytotoxicity with cold target and control cells were performed, using cytotoxic lymphocytes from two melanoma patients and from one healthy donor, at L/T ratios of 100 : 1, against 10^4 ^{51}Cr-labelled NK I_1 target cells, in CRT over a 20-hr incubation period. Cold NK I_1 cells, or fibroblasts, or lymphoblastoid cells LHN_{12}, were added in concentrations varying from 7×10^4 to 3×10^5 cells per millilitre. The cytotoxicity of lymphocytes from melanoma patients was partially decreased by cold NK I_1 cells, but also to a certain extent by fibroblasts. The cytotoxic effect of normal lymphocytes was also reduced by NK I_1 and fibroblasts, but not by LHN_{12}. These limited experiments therefore failed to provide evidence for a specific immune reaction.

The cytotoxicity of the same lymphocyte suspension from a 'cytotoxic' melanoma patient was tested in CRT using either SK Mel_1 melanoma cells or normal PHA-stimulated lymphocytes as target cells. The results showed that, at 2, 20 and 28 hr, the ^{51}Cr release from PHA blasts induced by the patients' lymphocytes was similar to that induced by control lymphocytes from a normal donor (M.O.L.) at the same L/T ratio (100 : 1). In contrast the ^{51}Cr release from SK Mel_1 induced by the patient lymphocytes at 20 and 28 hr was respectively 21% and 25% above that induced by control lymphocytes. PHA blasts may be less susceptible to cytolysis than SK Mel_1, but were readily lysed by HL-A-immune lymphocytes in positive controls.

DISCUSSION

The toxicity of the lymphocytes from melanoma patients and normal subjects has been studied here, using the Cr release test, against two melanoma target cell lines, one growing in monolayer (NK I_1) and one growing in suspension (SK Mel_1). The first notable result is the finding that leucocytes from healthy subjects are spontaneously toxic for the melanoma target cells in cultures. The lymphocyte cytotoxicity increases with the L/T ratio, varies from one individual to another, and some leucocyte suspensions from normal donors may be far more toxic than those from some melanoma patients.

The reasons for the cytotoxic effect of normal lymphocyte suspensions necessitates closer examination. It is unlikely to be simply due to a depletion of the nutrients of the reaction culture medium, or to the diluent used for lymphocyte purification, since some lymphocytes were highly toxic and others not under the same experimental conditions. The role of the freezing procedure in rendering the cell suspensions cytotoxic was eliminated by repeated experiments where frozen cells appeared even less toxic than fresh cells. Such a cytotoxicity *in vitro* of lymphocytes from normal donors against a variety of cultured tumour cells has already been reported in microcytotoxicity assays (Takasugi, Mickey & Terasaki, 1973; McKhann, Cleveland & Burk, 1973; Bloom, Ossorio & Brosman, 1974), which may reflect an inhibition of growth rather than a direct cytotoxic effect. Normal human lymphocytes were also reported to induce short-term membrane lesions on fresh (McCoy *et al.*, 1973) or cultured (Peter *et al.*, 1974; McCoy *et al.*, 1973) tumour target cells in ^{51}Cr release assays. We found some variations in the sensitivity of different melanoma cell lines to this cytotoxic effect (H. H. Peter and J. Pavie, unpublished observations).

Using three preparations of ^{51}Cr-labelled fresh melanoma cells derived from metastatic lymph nodes, we noted only minor if any increase in ^{51}Cr release in the presence of normal lymphocytes (unpublished results), suggesting that cultured cells may be more sensitive than fresh cells to the cytotoxic effect of normal lymphocytes.

Nothing allows us to state at present if the *in vitro* cytotoxicity of lymphocytes from healthy donors is immunological in nature, or due to a non-specific toxicity of one or several lymphocyte subpopulations. The responsibility of polymorphonuclear leucocytes can be eliminated in our experiments, since they are totally lacking from some toxic leucocyte suspensions. Several types of mononuclear cells are known to be potentially toxic for targets cells and include activated macrophages, T cells and killer cells mediating antibody-dependent cytotoxicity (provisionally called 'K' cells), which seem particularly effective in human blood (McLennan, 1972; Perlmann, Perlmann & Wigzell, 1972). In recent experiments (Peter *et al.*, 1975), we found that the toxic effect of normal human lymphocytes was mainly associated with non-T cells, presumably K cells, whereas the elimination of phagocytic and adherent cell series failed to suppress the cytotoxicity of lymphocyte suspensions. In the absence of well-defined immune reactions of normal donors against melanoma cell lines, one could speculate that some mononuclear cells, possessing a non-specific cytotoxic potential, such as K cells, may be selectively revealed using established target cell lines, because of an increased sensitivity of the latter cells to cell-mediated lysis. It would be important to determine if the cell population(s), toxic in normal subjects, are the same which act(s) as cytotoxic effectors in specific immune reactions *in vitro*.

When considering the whole series of melanoma patients, the lymphocyte cytotoxicity toward both cell lines was not significantly different from that observed in normal subjects. However, the more toxic among lymphocytes from melanoma patients concern patients with primitive and localized tumours, and not those with metastases. The variable cytotoxicity of lymphocytes from normal subjects makes it difficult to compare the toxic effect of patient's lymphocytes to that of normal subjects, as usually done in cell-mediated cytotoxicity assays *in vitro*, since the results of tests depend upon the unpredictable values found for normal controls. We tried to make such a comparison, however, using as 'normal' controls either a healthy subject taken at random, or a standard lymphocyte suspension from a 'non-toxic' donor, and it is noteworthy that all 'positive' reactions were found among patients with primitive and localized tumours. In fact, our experimental conditions show that the definition of what should be considered as a 'positive' cytotoxic reaction is probably less arbitrarily defined by comparison with a spontaneous ^{51}Cr release from target cells than by comparison with a variable level of cytotoxicity of normal lymphocytes.

The specificity for melanoma-associated antigens of the cytotoxic effect of lymphocytes from some melanoma patients could not be insured, although limited evidence is presented that patient's lymphocytes react more in CRT with a melanoma cell line than with PHA blasts. Since this ^{51}Cr release assay still lack an internal control for immunological specificity, it cannot be excluded that the different cytotoxic effects of lymphocytes from patients with primitive tumours and from patients with metastasis are due to a non-specific cytotoxicity of some mononuclear cells, more numerous in the peripheral blood at the onset of the disease, or blocked during the dissemination stage. Alternatively, a relatively high level of non-specific lymphocyte cytotoxicity may exist in the peripheral blood of melanoma patients, as in healthy subjects, and mask immune cytotoxic reactions, which presumably predominate in patients with primitive tumours, in the absence of metastasis.

It seems therefore that the ^{51}Cr release assays using long-term tissue culture lines as target cells reveal a significant cytotoxic effect of normal human lymphocytes. The use of this assay in the search for cell-mediated anti-tumour reactions in humans may require careful selection of the target cells and perhaps the use of purified T-lymphocyte suspensions, devoid of spontaneous cytotoxicity (Peter *et al.*, 1974b), provided that they include immune cytotoxic effectors active in ^{51}Cr release assays.

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