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The Interaction of Normal Lymphocytes and Cells from Lymphoid Cell Lines

I. THE NATURE OF THE ACTIVATION PROCESS

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Summary. Human peripheral lymphocytes from all donors tested were mitotically activated when cultured in the presence of X-irradiated cells from continuously cultivated lymphoid cell lines (LCL). X-irradiated cells from LCL originating from normal individuals were effective as well as those of Burkitt's tumour origin and cells from LCL reported to be free of herpes-like virus were as effective as those reported to contain virus. The activation was of much greater intensity than the normal mixed lymphocyte reaction but occurred over a similar culture period. The reaction occurred only under conditions permitting direct cell contact between the two populations of cells. Most stimulation was obtained with viable X-irradiated LCL cells. Disrupted LCL cells were ineffective.

INTRODUCTION

It is now well established that lymphocyte activation occurs in mixed cultures of lymphocytes originating from two unrelated donors. The reaction, which requires no prior immunization of the cell donors, may occur in mixed cultures of thymic, splenic or blood lymphocytes from any two unrelated donors within a species (see Hardy and Ling, 1969 and Ling, 1968 for references) and the intensity of the reaction in humans is related to differences at the HL-A locus of the cell donors (Amos and Bach, 1968; Schellekens *et al.*, 1970). A 'one-way' reaction (in contrast to the normal 'two-way' reaction) occurs when one of the donors is tolerant of the cells of the other or one population is rendered unresponsive to stimulation by X-irradiation or drug treatment (Schwarz, 1968; Kasakura and Lowenstein, 1968; Wilson, 1967; Bach and Voynow, 1966).

A stimulation of normal lymphocytes by cells from lymphoid cell lines (LCL) would be expected to occur because the continuously cultured lymphoid cells probably have on their surface most of the antigens present on normal lymphoblasts and resemble them in cultural characteristics. In addition many of the LCL contain a herpes-like virus which may, according to recent reports, have the capacity of inducing continuous proliferation of populations of normal lymphocytes (Henle, Diehl, Kohn, Hansen and Henle, 1967; Pope, Horne and Scott, 1968). We have found that there is indeed considerable activation of normal lymphocytes by X-irradiated cells from LCL which is of the order of ten times that of a normal mixed lymphocyte reaction (Hardy, Ling and Knight, 1969). The activation might be identical in type to that observed in normal mixed lymphocyte cultures, differing only in magnitude. It might be correlated with the presence of virus in the

LCL cells or it might be associated with the continuously dividing state of the LCL cells or to abnormal antigens on their surface. In this paper we have analysed the reaction process using modified cells and disrupted cells and various reaction conditions. In the following paper an antigenic analysis of the reaction is attempted by comparing the properties of lymphoid cells from various sources.

MATERIALS AND METHODS

Cultures of normal lymphocytes and lymphocyte mixtures

One-millilitre volumes of the cell suspensions in Eagle's medium containing 20 per cent human serum-gelatin were cultured in triplicate in loosely capped glass tubes 7·5 cm × 1·25 cm. The Eagle's medium (Glasgow modification) contained penicillin (200 units/ml) and streptomycin (100 units/ml) and was prepared in the Department of Virology of this University. Cultures were gassed with 5 per cent CO_2 in air.

Tritiated thymidine was obtained from the Radiochemical Centre, Amersham, at a specific activity of approx. 3000 mCi/mM thymidine-(methyl-T). This solution was diluted with 'cold' thymidine before addition to cultures to a specific activity of 150 mCi/mM; 0·5 μCi was added to cultures 24 hours before harvesting. Cultures were processed for scintillation counting or autoradiography as previously described (Knight and Ling, 1969). For scintillation counting cell deposits were washed once in saline, twice in 5 per cent trichloracetic acid and once in methanol. Cell deposits were then dissolved in N NaOH (0·1 ml) and transferred, with 10 ml of a xylene-based phosphor, to a counting bottle and counted in a Packard Tricarb Scintillation Counter. Cell deposits from some cultures containing high concentrations of red cells were washed in 1 per cent acetic acid in saline instead of saline and left for 2 hours at the methanol stage before centrifuging. Viability of cells was assessed by trypan blue exclusion. Cell numbers referred to throughout the paper are numbers of dye-excluding cells.

Lymphoid Cell Lines (LCL)

Many cell lines of a lymphoid character are now in continuous cultivation in laboratories in various parts of the world. The first cell lines were established from the cells of patients with a leukaemia or Burkitt's lymphoma but in recent years LCL have also been established from blood cells of normal individuals (see Moore and McLimans, 1968 for references). A gradual decline in the number of viable leucocytes typically occurs during the early phase of the culture and is followed, after a few weeks, if the culture 'takes', by a gradual rise in the numbers of mononuclear cells not attaching to glass.

The RPMI LCL were sent to us from Dr Moore's laboratory in suspension in bottles almost full of medium. The NHDL LCL were received in the frozen state from Dr Gerber. These LCL had been maintained in RPMI 1640 or McCoy's medium supplemented with human or foetal calf serum. Many Burkitt's lymphoma cell lines have been maintained in more traditional medium such as Eagle's medium supplemented with human or calf serum. In our experience the EB cell lines grow well in Eagle's medium supplemented with calf or human serum. The RPMI and NHDL cell lines may be maintained for short periods in this medium but require RPMI or McCoy's medium supplemented with foetal calf serum for continuous maintenance. Medium RPMI 1640 was obtained from Microbiological Associates. Foetal calf serum was obtained from Flow Laboratories Ltd.

The following cell lines were used:

Cell line derivation	*Source*	*Reference*
EB2 EB4 EB5	Prof. M. A. Epstein	Epstein, Barr and Achong, 1966
NHDL-1 NHDL-2 NHDL-3 NHDL-4	Dr P. Gerber	Gerber and Monroe, 1968
RPMI-5287 RPMI-6237 RPMI-7216 RPMI-7466	Dr G. Moore	Moore and McLimans, 1968: Moore (personal communication)

All these cell lines are broadly lymphoid in morphology and the cells do not attach themselves to glass surfaces but there were marked differences in mode of growth in our laboratory. EB4 cells, for example, grew diffusely whereas some of the NHDL lines grew in tight clumps.

X-irradiation of cells

LCL cells or normal lymphocytes were X-irradiated from a 200 kV source at 12·5 mA using an aluminium filter 1 mm thick. The dose rate was 150 R/min. During the irradiation the cells were present in culture medium at a concentration of 2×10^6 per ml in a universal container at room temperature.

Chromosome preparations

A modification of the Moorhead technique was used (Edwards and Young, 1961).

Lymphoid cell treatments

(a) *Aldehyde preserved cells* were prepared by treating lymphocytes with pyruvic aldehyde or glutaraldehyde under the conditions previously described for red cells (Ling, 1961) followed by washing in Eagle's medium.

(b) *Freeze-dried cells.* Cells of the EB2 or EB4 lines were spun down and resuspended in Eagle's medium (without serum) at a concentration of 200×10^6/ml. Small volumes were freeze-dried in ampoules.

(c) *Disrupted lymphoid cells.* Frozen-thawed preparations refer to lymphoid cells which had been frozen in an acetone icebath and thawed at 37°, the process being repeated five times. Sub-cellular fractions were prepared from LCL by a method used by Dr A. Sanderson to prepare transplantation antigens (Sanderson and Batchelor, 1967). $2{\cdot}5\times10^8$ cells were incubated at 4° in 10 ml of distilled water for 3 hours and the 'nuclear' and 'microsomal' fractions prepared by differential centrifugation.

(d) *Potassium chloride extracts.* 20×10^6 EB2 cells were pelleted and taken up in 2 ml of 11 per cent KCl. The preparation was centrifuged at 9000 ***g*** and the deposit washed once in Eagle's medium and then taken up in Eagle's medium+serum.

(e) *Urea treatment.* 20×10^6 EB2 cells were spun down and taken up in 2 ml of 36 per cent (6 M) urea. The cell coagulum was spun down at 900 ***g***, washed once in Eagle's medium, and taken up in Eagle's medium+human serum (2 ml).

(f) *Acetone treatment.* 5×10^7 EB2 cells were taken up in a small volume of Eagle's medium and shot into 20 ml of cold acetone. After centrifugation the cell deposit was washed once with Eagle's medium and taken up in Eagle's medium+human serum (2 ml).

(g) *Ethylene diamine tetracetate (EDTA) extraction.* The cells were treated with EDTA by a technique adapted from that of Beierle (1968) which he used for the extraction of a factor enhancing cellular proliferation from polyoma virus-transformed BHK-21 cells. EB5 cells (51×10^6) were spun down, washed once in 0·02 per cent EDTA (disodium salt) in saline (20 ml) and then taken up in 2 ml of the same solution. The preparation was left at 4° overnight and then centrifuged at 900 ***g*** for 30 minutes. The cells were resuspended in fresh EDTA-saline and left at 4° for various periods. Both cells and supernates were tested.

(h) *Culture supernates.* Supernates of LCL cultures were prepared by simply spinning out the cells at various times after setting up the cultures in fresh medium. Concentrated supernates were prepared as follows: (1) 86×10^6 EB2 cells were spun down and taken up in Eagle's medium (40 ml). After 2 hours the cells were spun down and taken up in Eagle's medium without serum (17 ml) and left 16 hours at 37°. The supernate from a low speed 900 ***g*** spin was recentrifuged at 9000 ***g*** to clear it completely of cellular debris and this supernate was frozen at −20° until tested. The cells were resuspended in Eagle's medium and stored at 4° for periodic testing of stimulatory capacity. It was discovered during the course of these experiments that EB2 cells survive for long periods in Eagle's medium lacking serum either at 4° or at 37°. (2) Culture supernates similar to that just described were stored frozen then thawed and then pooled and after dialysis against distilled water were freeze-dried. The dry deposit was reconstituted in Eagle's medium+serum to 1/10th the original volume of the culture.

(i) *Millipore chambers.* Millipore membranes (0·22 μ pore size) were cemented onto polythene tubes (0·5 cm diameter) and sterilized by high doses of X-irradiation. X-irradiated NHDL-2 cells (10^6) were introduced to the chamber, which was placed in a $3 \times \frac{1}{2}$ in. tube containing 10^6 normal lymphocytes so that the open end was well above the level of the liquid. The total volume was 1 ml. The cells were cultured for 144 hours 0·5 μCi ^{3}H thymidine was added 24 hours before harvesting. The control consisted of a mixture of the irradiated NHDL cells and normal lymphocytes cultured each side of the membrane under the same conditions. At harvesting the cells were washed from the membrane with saline, added to the cells in the tubes and prepared for liquid scintillation counting in the usual way.

Human peripheral lymphocytes

Lymphocytes were harvested from defibrinated human blood sedimented with gelatin by the method of Coulson and Chalmers (1964). The cells were spun down and resuspended in Eagle's medium containing 20 per cent pooled human serum-gelatin to a leucocyte concentration of 2×10^6 per ml.

Lymphocyte mitogens

Phytohaemagglutinin batch X5 was a gift from Burroughs Wellcome & Co. Ltd. It was very active against human blood lymphocytes at a concentration of 2 μg/ml. Staphylococcal filtrate batch 20 was prepared as previously described (Ling, Spicer, James and Williamson, 1965). It was very active against human blood lymphocytes at a concentration of 1 in 20.

EXPERIMENTS AND RESULTS

KINETICS OF THE REACTION

The conditions sought for the X-irradiation of the LCL cells were those which would prevent cell division and reduce the capacity to incorporate tritiated thymidine to a low level by day 3 or day 4 while maintaining for as long as possible the viable state of a substantial part of the cell population. As can be seen from Table 1 and Fig. 1 a dose of between 2000 and 8000 R was required for prompt mitotic arrest of EB4 and EB2 cells and

TABLE 1

THE SURVIVAL OF X-IRRADIATED LCL CELLS

Cell line	Irradiation dose (R)	Hours after X-irradiation						
		−2	1	24	48	72	120	168
EB4	500	0·58	0·58	0·65	1·1	1·0	0·88	0·12
EB4	1000	0·58	0·61	0·61	0·65	0·59	0·21	0·06
EB4	2000	0·58	0·61	0·64	0·38	0·44	0·13	0·09
EB4	4000	0·58	0·59	0·44	0·30	0·40	0·13	0·05
EB4	8000	0·58	0·58	0·49	0·28	0·22	0·06	0·05
EB2	5000	0·53	0·55	0·82	0·65	0·70	0·75	0·54
EB2	1000	0·53	0·53	0·34	0·50	0·48	0·38	0·20
EB2	2000	0·53	0·53	0·44	0·45	0·46	0·24	0·11
EB2	4000	0·53	0·52	0·34	0·31	0·36	0·19	0·07
EB2	8000	0·53	0·53	0·33	0·18	0·19	0·06	0·04

The figures entered refer to millions of dye-excluding EB cells. Cultures were in 1 ml volumes in tubes. Half the medium was removed from the tubes at 72 hours and 144 hours and replaced by fresh medium.

6000 R was routinely used for irradiation of all LCL cells. At the lower dose levels the cells continued to multiply for several days producing exhaustion of the medium. It was necessary to change part of the medium thereafter to maintain survival. At the higher dose

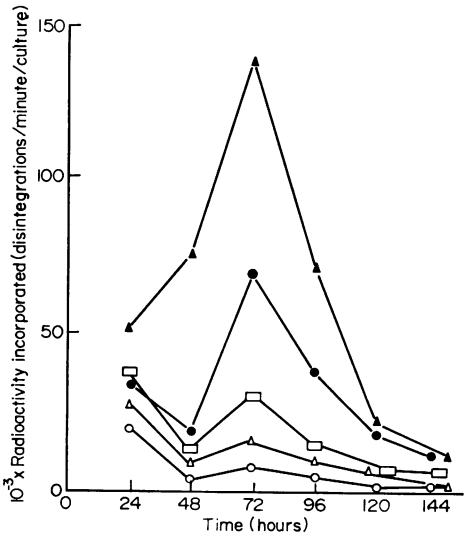

FIG. 1. The effect of different doses of X-irradiation on tritiated thymidine incorporation by lymphoid cell line cells. EB4 cells were X-irradiated (500 R ▲ ; 1000 R ● ; 2000 R □ ; 4000 R △ or 8000 R ○) and cultured for the times indicated. The initial cell concentration was 5×10^5 cells/ml; 0·5 μCi tritiated thymidine was added 24 hours before harvesting. Points are means of triplicate cultures.

TABLE 2

THYMIDINE INCORPORATION AND BLAST CELLS PRESENT IN MIXED CULTURES OF IRRADIATED EB2 CELLS AND HUMAN LYMPHOCYTES

Days of culture	Composition of culture	Per cent blast cells (smears)	Per cent labelled cells (autoradiographs)	^{3}H-thymidine incorporation (disintegrations/min)
2	L(10^6)+E (10^5)	16	16	7280
	L (2×10^6)	2	2	784
	E (2×10^5)	–	–	3037
4	L(10^6)+E(10^5)	48	33	46,880
	L(2×10^6)	3	1	4265
	E (2×10^5)	–	–	1675
7	L (10^6)+E (10^5)	61	39	108,046
	L (2×10^6)	1	12	21,130
	E (2×10^5)	–	–	330
8	L (10^6)+E (10^5)	11	26	46,422
	L (2×10^6)	4	17	22,204
	E (2×10^5)	–	–	351
9	L (10^6)+E(10^5)	18	–	15,192
	L (2×10^6)	1	–	9684
	E (2×10^5)	–	–	433

L: fresh blood lymphocytes from a normal human donor. E: X-irradiated EB2 cells.
Autoradiographs and scintillation counts refer to cultures to which ^{3}H-thymidine was added 24 hours prior to harvesting.

levels about 5–10 per cent of the cells survived for 6 or 7 days. Some of the surviving cells were grossly abnormal morphologically and many multinucleated cells were seen but there were also many cells indistinguishable from those seen in cultures not irradiated.

The optimal concentration of X-irradiated LCL cells for maximal stimulation of the incorporation of tritiated thymidine by irradiated LCL-normal lymphocyte mixtures was not the same for all the cell lines tested. More activity was consistently found in mixtures containing 10^6 EB4 cells/ml than in those containing 10^5/ml whereas the reverse was true of EB2. The limiting concentration was related to the usage of medium over the culture period by the actively metabolizing irradiated EB cells. At a concentration greater

TABLE 3

LYMPHOCYTE STIMULATION WITH DIFFERENT CONCENTRATIONS OF IRRADIATED EB4 CELLS

Days of culture	No. of irradiated EB4 cells present		
	10^6	10^5	10^4
	^{3}H-thymidine incorporation*		
4	28,761 (21,141)	10,513 (5786)	1007 (-ve)
6	114,635 (109,661)	23,059 (19,320)	3065 (647)
8	96,012 (92,500)	5580 (2792)	3489 (896)
11	31,933 (28,643)	3564 (1537)	2009 (-ve)

All cultures contained 10^6 lymphocytes from a normal donor.

* Tritiated thymidine incorporated into the acid-insoluble fraction disintegrations/min during the last 24 hours of the culture period. The first figure represents mean disintegrations/min of triplicate cultures and the bracketed figure the increment of stimulation (disintegrations/min mixed cultures minus half the sum of the disintegrations/min control, unmixed cultures).

TABLE 4

LYMPHOCYTE STIMULATION WITH DIFFERENT CONCENTRATIONS OF IRRADIATED EB2 CELLS

Donor of normal lymphocytes (10^6)	Days of culture	No. of irradiated EB2 cells present 10^5	$0{\cdot}5 \times 10^5$	$0{\cdot}25 \times 10^5$
		^{3}H-thymidine incorporation*		
J.L.	5	118,402 (117,952)	86,935 (86,485)	59,210 (58,760)
D.H.	3	40,447 (39,155)	–	–
D.H.	4	87,015 (85,923)	–	–
D.H.	5	144,280 (142,638)	–	–
D.H.	6	150,809 (148,563)	–	–

* Calculated as for Table 3.

than $0{\cdot}5 \times 10^6$/ml irradiated EB2 cells produced a pronounced fall in the pH of the medium the colour changing to yellow by the 4th day. In most experiments 10^6 normal lymphocytes were present in the 'mixture' tubes and 2×10^6 in the 'control' tubes but in some experiments controls were also set up at 1×10^6/ml. There was little difference in the background correction at these two concentrations.

The elevated incorporation of tritiated thymidine measured by scintillation counting was accompanied by an increase in the number of blast cells in smears and in the number

TABLE 5

TWO-WAY MIXED NORMAL LYMPHOCYTE REACTION

Cell donors	Days of culture	^{3}H-thymidine incorporation*
M.+L.	5	6152 (5454)
M.+B.	5	2558 (2077)
M.+S.	5	2553 (2193)
L.+B.	5	7725 (6837)
L.+S.	5	4496 (3729)
B.+S.	5	1855 (1455)
W.+L.	5	3502 (2910)
W.+L.	6	7525 (6765)
W.+L.	7	8481 (7370)
H.+C.	8	991 (885)
H.+C.	9	4777 (4157)
H.+C.	10	7505 (6882)
H.+C.	11	7277 (6453)

*Calculated as for Table 3. Mixed cultures contained 10^6 cells from each donor.

of labelled cells present in autoradiographs (Table 2). The reaction was a slow one, the maximum not being reached until day 5 or later (Tables 3 and 4). Mitotic figures were seen at this time.

The level of activation in normal lymphocyte+irradiated LCL mixtures was much greater than that occurring in two-way mixed normal lymphocyte reactions (Table 5).

ONE-WAY NATURE OF THE STIMULATION

A small increment of activity above 'control' values was found in cultures in which the normal lymphocytes as well as the LCL cells were irradiated (Table 6). However, the increment was only a small fraction of that found in mixtures containing irradiated LCL cells and untreated normal lymphocytes and might simply be a cell concentration effect. This result strongly indicated that in the standard cultures the normal lymphocytes were being stimulated by the irradiated LCL cells and were not in some way rejuvenating the irradiated LCL cells. This impression was confirmed by the morphological observations and particularly by the numbers of blast cells present. Chromosome preparations were examined. All mitotic figures seen were in the normal range and lacked the distinctive markers found in LCL chromosome preparations or chromosome breaks resulting from X-irradiation indicating that the dividing cells originated from the normal lymphocyte population.

TABLE 6

UNRESPONSIVENESS OF IRRADIATED LCL CELLS

Cells or reagent added to irradiated LCL cells	X-irradiated LCL cells	Days of culture	^{3}H-thymidine incorporation*
10^6 normal lymphocytes	10^6 EB4	5	101,696 (97,495)
10^6 X-irradiated normal lymphocytes	10^6 EB4	5	4189 (3397)
10^6 normal lymphocytes	10^5 RPMI-1737	5	151,305 (147,661)
10^6 X-irradiated normal lymphocytes (1000 R)	10^5 RPMI-1737	5	6673 (6403)
Staphylococcal filtrate (1 in 10)	10^6 EB4	4	4186 (83)
filtrate (1 in 10)		6	2348 (4)
Phytohaemagglutinin (2 μg/ml)	10^6 EB4	4	3851 (321)
Staphylococcal	10^6 EB2	2	2212 (463)
filtrate (1 in 10)		3	1261 (−247)
Phytohaemagglutinin (2 μg/ml)	10^6 EB2	2	6414 (1976)
		3	4919 (963)
Human red cells (20×10^6)	10^6 EB2	5	2189 (−787)

* Calculated as for Table 3.

Red cells alone had no effect on the survival or responsiveness of isolated LCL cells but slightly reduced the activity of the LCL-normal lymphocyte mixtures as judged by scintillation counting (Table 5). The possibility that this apparent inhibition was due to a counting artefact was eliminated by using the modified processing procedure to remove haemoglobin.

Role of virus in the activation

The degree of activation obtained with the various LCL did not appear to be related to the presence of herpes-like virus in the cell line. One of the most active lines (EB2) was one in which virus has been readily demonstrated by others but cells from LCL said to be free of virus were not less active in general than those known to be infected by virus.

Antibodies to EB virus antigens are known to be common in human sera and were presumably present in the various pools of normal human sera used in the cultures. Irradiated LCL cells were as active as stimulants even at very low concentrations (Table 6) and there were no indications from dose–response curves of the presence of 'blocking' antibodies relevant to this activation. No significant 'blocking' of the stimulatory activity of irradiated EB4 cells was found when these cells were absorbed with a large volume of serum from individuals recently recovered from infectious mononucleosis.

Stimulation with irradiated LCL cells did not produce more than a temporary improvement in the survival of the normal lymphocytes. By day 9 the cultures of normal leucocytes to which irradiated EB cells had been added were indistinguishable, in terms of numbers of viable cells, from control cultures. The cell survival rate was similar to that found in a normal mixed lymphocyte reaction.

The highest levels of stimulation of normal lymphocytes by irradiated EB cells were obtained when the EB cells were harvested from a well maintained culture containing cells dividing at the maximal rate. Cells from 'starved' cultures were not as effective.

STIMULATORY ACTIVITY OF CELL PRODUCTS

Various alternative methods of killing or arresting growth of LCL cells were explored and the activities of whole cells, broken cells and substances released by the cells into the culture medium were tested. The results are summarized in Tables 7 and 8. Freezing and thawing the LCL cells several times destroyed their stimulatory potential. Preparations of LCL frozen and thawed only once retained some activity but this could be accounted for by the fact that some intact and dye-excluding cells were found in these preparations. Freeze-dried cells (either the whole preparation or the cell ghosts obtained after washing) were also inactive even though little disruption of gross cell structure had occurred. Cells treated with urea or acetone or aldehydes were also inactive. There was slight and possibly significant activity in preparations of cells treated with strong KCl.

Tests for diffusible activating substances were made on cell-free culture supernates or concentrates of culture supernates prepared after growing the cells in serum-free medium by separating the irradiated LCL cells and the normal lymphocytes by a Millipore membrane (Table 8). Some apparent stimulation was occasionally obtained with culture supernates but it was always of a low order and as activity was not found in concentrated supernates it may have been simply due to small molecular metabolites exerting a 'feeder' effect. The Millipore experiments excluded the possibility that labile diffusible substances had any primary role in the mixed cell activation. There did not appear to be any blastogenic substances on the LCL cells which could be released by incubation with EDTA.

Subcellular fractions of LCL cells prepared by the method of Sanderson and Batchelor (1967) were not significantly active. The 'microsome' fraction was inhibitory and although there was some activity in the 'nuclear' fraction this could be accounted for by the residue of unbroken cells in this fraction (Table 7).

TABLE 7

ACTIVITY OF LCL CELL PREPARATIONS AND LCL CELL FRACTIONS

Preparation added to culture	Days of culture	^{3}H-thymidine incorporation*
Irradiated EB4 cells (10^6)	5	88,950 (83,950)
EB4 cells (10^6) frozen and thawed 4 times	5	3571 (negative)
EB4 cells (10^7) frozen and thawed 4 times	5	3672 (negative)
Irradiated EB4 cells (10^6)	9	13,165 (8653)
EB4 cells (10^6) frozen and thawed 4 times	9	9317 (5307)
EB4 cells (10^7) frozen and thawed 4 times	9	12,480 (7475)
Irradiated EB2 cells (2×10^5)	5	95,703 (92,888)
Freeze-dried EB4 cells (2×10^6)	5	2548 (nil)
Freeze-dried EB4 cells (2×10^6) washed	5	3508 (693)
KCl-treated EB2 cells (2×10^6)	5	6691 (3876)
KCl-treated EB2 cells (2×10^5)	5	4606 (1791)
Urea-treated EB2 cells (2×10^6)	5	3894 (1079)
Urea-treated EB2 cells (2×10^5)	5	3641 (826)
Acetone-treated EB2 cells (2×10^6)	5	2852 (37)
Irradiated EB4 cells (10^6)	6	12,916 (10,516)
EB4 cells (10^6) preserved with pyruvic aldehyde	6	914 (52)
EB4 cells (10^6) preserved with glutaraldehyde	6	420 (−240)
Irradiated EB4 cells (10^5)	6	95,282 (87,877)
EB4 cells (10^7) 'nuclear' fraction	6	13,450 (5790)
EB4 cells ($3{\cdot}7 \times 10^7$) 'microsome' fraction	6	4273 (negative)

* Calculated as for Table 3. All cultures listed contained 10^6 viable lymphocytes from a normal human donor.

DISCUSSION

X-irradiation of cells is universally recognized as a means by which the metabolic integrity of the cell may be minimally disrupted while arresting mitotic activity. It has been used for producing one-way reactions in the mixed lymphocyte reaction (Kasakura and Lowenstein, 1968). Small lymphocytes are unusually radiosensitive in comparison with other non-dividing cells but become more radioresistant when they are activated to a blast state (Schrek, 1968). The dose–survival curves of LCL cells subjected to X-irradiation appear to be similar to those of cells of other continuously dividing cell lines. Division delay is known to increase with irradiation dose and at very high dose levels the surviving cells rarely divide even once but continue metabolizing and may enlarge to giant cells (Little, 1968). The LCL cells conformed to this pattern after irradiation. After 6000 R

TABLE 8

ACTIVITY OF CULTURE SUPERNATES

Origin of supernate	^{3}H-thymidine incorporation*		
	Concentration	test	Irrad. cells (pos. control)
24 hour EB2 culture	40%	17,924 (12,955)	151,988 (122,500)
24 hour NHDL4 incubation (6×10^7/ml)	50%	negative	155,249 (150,153)
incubation (6×10^7/ml)	3%	18,584	155,249 (140,153)
EB2 cells (5×10^6/ml) 24 hours at 37°		7,665	
72 hours at 4°	50%	(3,456)	–
72 hours at 4°	10%	3,811 (negative)	–
72 hours at 4°	1%	3,744 (negative)	–
EB5 cells (25×10^6/ml) in EDTA-saline, 24 hours at 4°	10%	4,200 (negative)	99,493 (95,282)
separated from irradiated NHDL2 cells by Millipore		1,280 (negative)	38,795 (36,917)

* Calculated as for Table 3. Cultures listed contained $1–2 \times 10^6$ viable lymphocytes from a normal human donor. Culture period = 5 days.

the numbers of surviving cells fell off over a period of days but 5–10 per cent survived the whole culture period.

There does not seem to be any doubt that the very high level of tritiated thymidine incorporation found in mixtures of X-irradiated LCL cells and normal lymphocytes is entirely accounted for by mitotic activation of the normal lymphocyte population. The number of blast cells present, the normality of the mitotic figures and the total lack of response of irradiated LCL cells to irradiated normal lymphocytes or to the lymphocyte mitogens phytohaemagglutinin and staphylococcal filtrate establish this fact. We have next to consider to what extent the activating property of irradiated LCL cells is viral, metabolic or antigenic in origin.

Some recent reports attach great importance to a lympho-proliferative role of herpes-like viruses. Following the demonstration of herpes-like viruses in LCL established from cells of patients with Burkitt's lymphoma (Epstein, Barr and Achong, 1964) and later in LCL established from the blood of normal individuals (Moore and McLimans, 1968) other evidence appeared linking a virus of this type with the lymphocyte proliferation seen in infectious mononucleosis (Pope, 1967; Henle *et al.*, 1968; Gerber, 1968). A report that continuous proliferation of normal human lymphocytes had been induced by transfer of virus from infected EB cells seemed to provide a further link in the chain of evidence (Diehl, Henle, Henle and Kohn, 1968). The properties and antigenic structure of the LCL cells may well be influenced by the presence of virus but there are a number of reasons why we are disinclined to believe that virus plays an important part in the activation we observe. Firstly the activation begins very soon after setting up the cultures and is of short duration, comparable to that seen in a normal mixed lymphocyte reaction. This contrasts with the activation induced by virus, the effects of which are not observed until some weeks after the introduction of virus and are maintained for long periods (Diehl *et al.*, 1968; Pope *et al.*, 1968). Secondly, disrupted cells from virus-containing LCL are ineffective whereas whole

irradiated cells from LCL reported to a be free of virus are active. Thirdly, populations of LCL cells which are dividing at a maximal rate appear to be the most effective as stimulants. Virus is hard to detect in maximally dividing LCL cells whereas the virus content of LCL cells is markedly increased when cell growth is slowed as in a nutritionally deficient medium (Henle and Henle, 1967). Our results indicate that it is the LCL cells themselves, and not the virus they may sometimes contain, which are potent activators of human small lymphocytes. Infection of the transformed lymphocytes by any virus present in the LCL cells could then be a likely subsequent event in view of the known capacity of lymphoblasts, in contrast to small lymphocytes, to support the growth of viruses (Knight and Najera, 1969). The experiments of Henle *et al.* (1967) could be reinterpreted on these lines. Our results are not, however, at variance with the evidence suggesting that viruses may have an initiatory role in lymphoproliferative states. If a single lymphoid cell capable of autonomous growth arose in the body through viral transformation and grew into a clone of cells these cells might, in turn, provoke an intense proliferation of normal lymphocytes.

Antibodies which react with constituents of cultured LCL cells are common in human sera. Some of these antibodies have been shown conclusively to react with herpes-type viral antigens (Henle and Henle, 1967; Old *et al.*, 1966), others to react with antigens on the membranes of cells of LCL containing the virus (Klein *et al.*, 1968). A high proportion of sera of patients recovering from infectious mononucleosis have been shown to contain antibodies to antigens of herpes-like virus (Gerber, Hamre, Moy and Rosenblum, 1968; Henle *et al.*, 1968). We have not observed any 'blocking' effects due to antibodies in the pools of normal human serum used in our cultures even when very low concentrations of irradiated LCL cells were used. Nor could blocking antibodies be demonstrated in our system in the sera of patients convalescing from infectious mononucleosis. Human sera also commonly contain antibodies which react with unidentified antigens in cells from LCL of various sources including those not containing virus (Herberman and Fahey, 1968). This report, taken together with the intense reaction of normal lymphocytes to LCL cells described in this paper, may be an indication that immunological reactions against these cells do occur in the body.

The need for intact viable LCL cells for anything like maximal stimulation of normal lymphocytes to occur exactly parallels our findings in relation to the normal mixed lymphocyte reaction (Hardy and Ling, 1969). Even minimally damaged freeze-dried LCL cells are inactive as are aldehyde-fixed intact cells whereas metabolically active cells incapacitated by X-irradiation were very active and intact cells heated to produce delayed death showed some stimulatory activity in spite of the fact that heating may have damaged some of the surface antigens.

There have been several reports that cultured leucocytes release antigenic substances or cell metabolites into the medium which are capable of activating small lymphocytes (Imrie and Mueller, 1968; Kasakura and Lowenstein, 1967, 1968). Direct tests on supernates of LCL cells indicated that substances liberated into the medium had only a very minor, if any, role in the activation of normal lymphocytes observed in our mixed cell cultures. This was confirmed by experiments in which the two populations of cells were separated by a Millipore membrane. As in the normal mixed lymphocyte reaction direct cell–cell contact between the two populations appears to be necessary. This does not exclude the possibility that mitogenic substances are released into the medium in the mixed cell cultures.

Cell–cell contacts are known to play an important part in antigen-induced activations

of small lymphocytes. This is so in activations produced by soluble antigens when cells which have taken up the antigen are thought, by surface contact, to activate those which have not ('recruitment'). In mixed lymphocyte reactions, too, crowding of cells together enhances activation by increasing the chances of surface interaction (Moorhead, Connolly and McFarland, 1967). Activation might also occur non–specifically by cell–cell interactions whether or not antigenic differences are involved. We are still uncertain about the extent to which the observed interactions of single cells with each other involve the transfer of information or equilibration of metabolic status. Highly active cells in a population might well activate resting cells of the same type even though they are genetically identical ('contagious activation'). In this type of activation direct transfer of cytoplasmic material from active to resting cells via intercytoplasmic bridges could possibly initiate the process. It is this distinction between antigen-induced and non-specific activation which has presented the greatest difficulties of interpretation and has prompted the experiments described in the following paper.

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