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The Interaction of Normal Lymphocytes and Cells from Lymphoid Cell Lines

II. VARIATIONS RELATING TO CELL LINE SOURCE AND LYMPHOCYTE DONOR

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Summary. Irradiated LCL cells from several sources activated the blood lymphocytes of a panel of donors. Individual differences were present but were small. Stimulated blood lymphocytes were more potent activators (after X-irradiation) than small lymphocytes in a one-way mixed lymphocyte reaction, indicating that the state of metabolic activity of the 'stimulating' lymphocyte affects the level of activation achieved. However, LCL cells incubated for several days after irradiation had not lost stimulatory capacity. Lymphocytes of pigs, rabbits, rats and mice were much less responsive than human lymphocytes. The response of animal lymphocytes generally varied with the species of origin of the serum used in the cultures.

INTRODUCTION

The fact that the marked stimulation of normal human lymphocytes by LCL cells completely disappears when the LCL cells are disrupted (see preceding paper) is in no way incompatible with an antigen-based stimulation. There are many indications that antigens on live cells are peculiarly effective in immunological reactions. For example, tumour immunity in mice may most easily be produced with live tumour cells (Alexander and Hamilton-Fairley, 1968). Antigens on peritoneal exudate cells are more immunogenic than the same antigens free in solution (Mitchison, 1969). Humphrey (1967) suggests, from a consideration of the methods by which delayed hypersensitivity is produced, that stimulation of lymphocytes is most effective when the antigen is exposed at the surface of a cell. Thus, as in the normal mixed lymphocyte reaction, the LCL-induced stimulation of normal lymphocytes may have an antigenic basis but occur only when the antigens are on viable cells. On the other hand, it is quite possible that lymphocytes may be activated by metabolically active neighbours even when antigenic differences may not be involved. For example, rat lymphocytes grown on embryo monolayers from the same rat strain have been reported to undergo activation (Ginsburg and Lagunoff, 1968). We have accordingly studied how the LCL-induced activation of normal lymphocytes is modified by variation of the antigenic and metabolic relationship of the two populations of cells.

MATERIALS AND METHODS

Lymphoid cell lines and conditions of cell culture, irradiation and preservation were as described in the previous paper.

Pig lymphocytes

Pig blood was defibrinated at the slaughter house. After the addition of a one-third volume of a 3 per cent gelatin the blood was allowed to sediment at 37° for 1–2 hours. The supernatant fluid usually contained $3–10 \times 10^6$ viable leucocytes per ml of which approximately 85 per cent were lymphocytes, 10 per cent neutrophils and 5 per cent eosinophils.

Rabbit lymphocytes

These were obtained from defibrinated blood by a similar technique. Approximately $0{\cdot}5–2{\cdot}0 \times 10^6$ viable leucocytes per ml were present in the supernates, the majority of which were lymphocytes.

Mouse and rat spleen cell suspensions

Spleens were disrupted by a single pressing in a glass homogenizer, and cell suspensions were filtered through nylon mesh. About 75 per cent of the cells were viable as judged by dye-exclusion.

Foetal calf serum was obtained from Flow Laboratories, Irvine, Scotland.

Antisera to LCL cells

Rabbits were injected repeatedly i.v. with the appropriate LCL cells in saline.

HL-A groupings

These were performed by Dr A. Sanderson, McIndoe Memorial Research Laboratories, East Grinstead, Sussex, and by Dr Van Rood, Leiden. The results are summarized in Table 1.

TABLE 1

SEROLOGICAL CLASSIFICATION OF LCL CELLS AND CELL DONORS

	HL-A nomenclature								Other tests positive	
Cells	1	2	3	4	5	6	7	8	Van Rood	Batchelor
N.L.	–	–	–	–	–	+4	–	–	6b	Gill, Trounce Huthwaite+
D.H.	++++	++	–	++++	–	–	–	–		Gill, Coster
S.T.	–	?	–	–	++	–	–	–		Gill
EB4	+++	–	–	++	–	+++	–	+++		
NHDL2	–	–	–	+++	–	+++	–	?		
NHDL4	+++	–	–	+++	+++	++	–	–		

The results refer to tests performed by Drs Van Rood and Sanderson. N.L., D.H. and S.T. were donors of fresh blood lymphocytes. EB4, NHDL2 and NHDL4 are lymphoid cell lines.

EXPERIMENTS AND RESULTS

STIMULATION IN RELATION TO LCL SOURCE

The EB lines EB2, EB4 and EB5 were tested systematically against a small panel of donors. A marked enhancement of tritiated thymidine incorporation usually occurred in cultures containing mixtures of X-irradiated EB cells and normal human lymphocytes compared with either population alone (Figs 1 and 2). There was a tendency for the same

FIGS 1 and 2. Increments of stimulation of the blood lymphocytes of various individuals by X-irradiated LCL cells (EB2, EB4 or EB5). Mixtures were cultured for 4 or 7 days as indicated, tritiated thymidine being present for the last 24 hours of culture. Results are corrected by subtraction of control values (lymphocytes or irradiated LCL cells cultured separately). The first block in each group of three refers to a mixture containing 10^6 irradiated LCL cells, the second 10^5 and the third 10^4. All cultures contained human serum from the same pool.

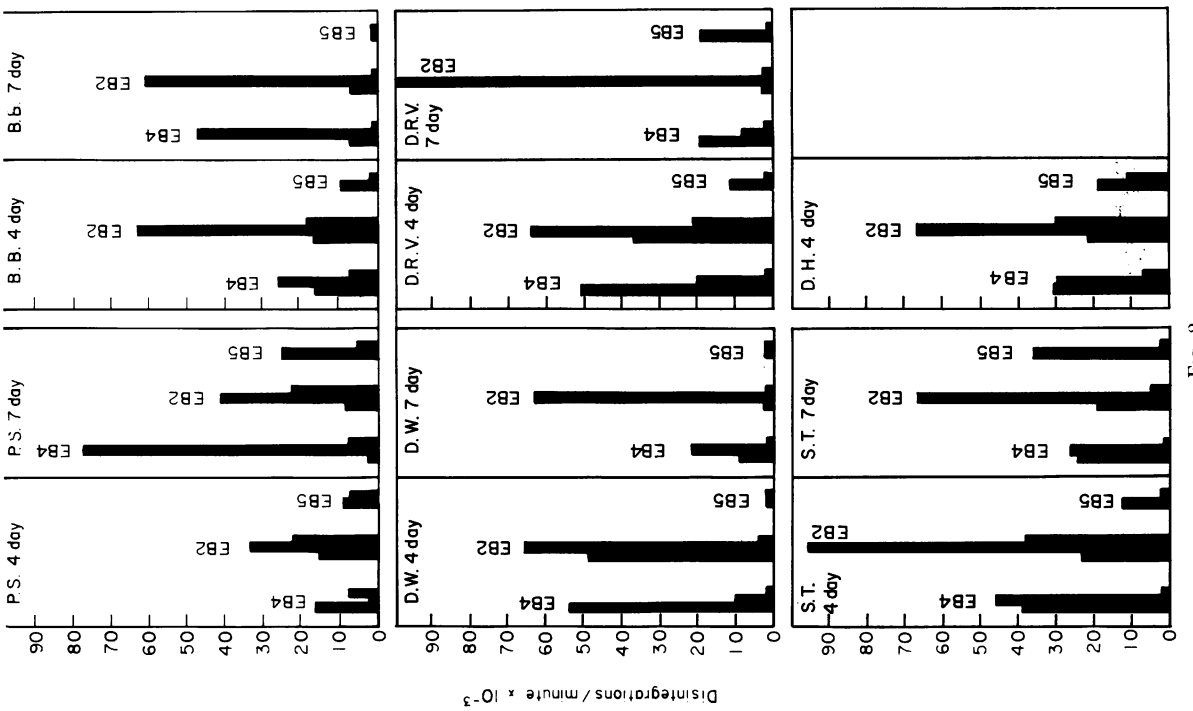
Disintegrations / minute x 10-3
P.S. 4 day
P.S. 7 day
D.W. 4 day
D.W. 7 day
D.R.V. 4 day
D.R.V. 7 day
B.B. 4 day
B.b. 7 day
S.T. 4 day
S.T. 7 day
D. H. 4 day
EB4
EB2
EB5
0
10
20
30
40
50
60
70
80
90

FIG. 2.

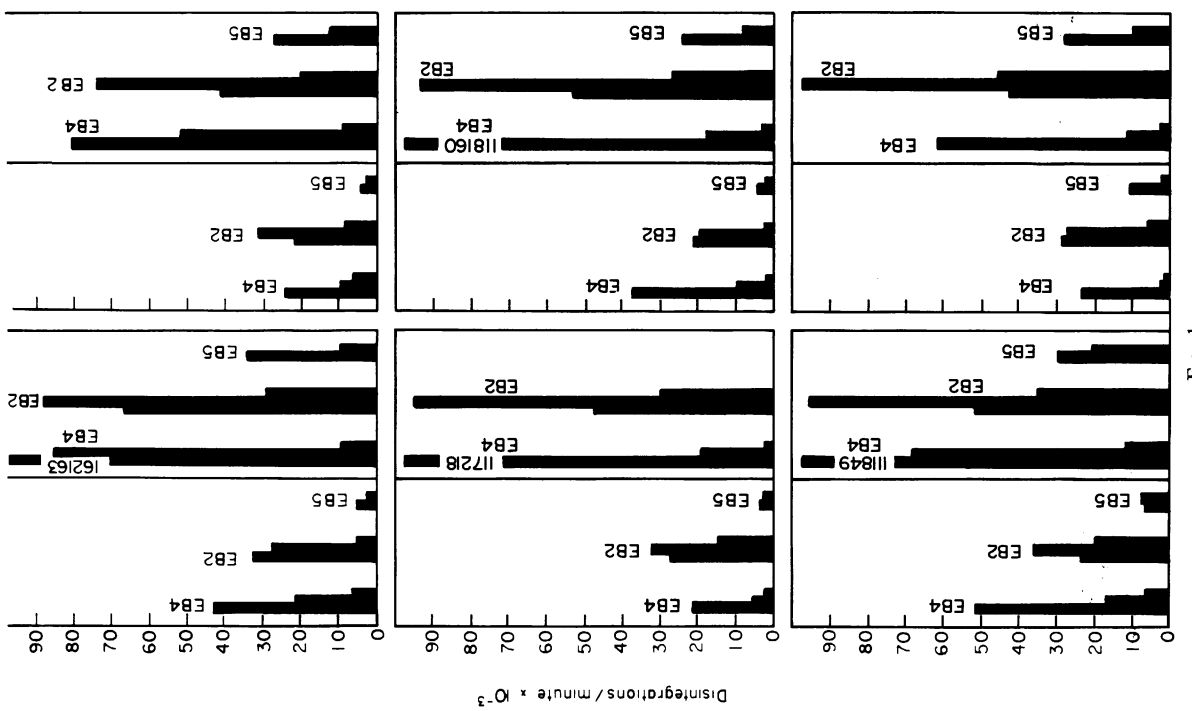
Disintegrations / minute x 10-3
162163
117218
118160
118449
EB4
EB2
EB5
0
10
20
30
40
50
60
70
80
90

FIG. 1.

pattern of activity relative to the three EB lines to be repeated with lymphocytes of different donors but there were some exceptions (e.g. R.K. in Fig. 2). There was a marked dose dependence of the EB cell stimulation although the optimum number of cells varied somewhat on different occasions depending perhaps on the growth rate of the LCL cells at the time of irradiation. This factor limited useful comparisons between cell donors to cultures set up at the same time. In the two experiments shown in Fig. 1 and Fig. 2 the activity in mixtures containing irradiated EB5 cells was low whereas on other occasions the activity has approached that in mixtures of irradiated EB4 cells and normal lymphocytes.

TABLE 2

EFFECT OF POST IRRADIATION INTERVAL ON STIMULATING CAPACITY OF EB2 CELLS

EB2 cells			^{3}H-thymidine incorporation* of mixtures containing cells irradiated on:				
No. viable when irradiated	? washed before mixing	Duration of mixed culture (days)	day −14	day −7	day−3	day −1	day 0
10^6	no	5	—	—	151,988 (144,234)	216,636 (208,522)	210,797 (202,853)
10^5	no	5	—	—	68,320 (63,238)	63,333 (58,331)	48,916 (43,878)
10^6	yes	5	—	—	131,393 (121,999)	136,977 (128,164)	—
10^5	yes	5	—	—	39,122 (33,974)	32,100 (27,037)	—
10^5	no	5	17,187 (14,901)	25,515 (23,218)	—	44,815 (41,651)	31,927 (29,056)

* Calculated as for Table 3 in previous paper. Mixed cultures contained 10^6 lymphocytes freshly isolated from the blood of a normal donor and X-irradiated EB2 cells.

COMPARISON OF FRESH AND 'INCUBATED' LCL CELLS

There was surprisingly little loss of stimulatory activity during post-irradiation incubation of LCL cells. Cells cultured for some days after irradiation were almost as active as cell suspensions used immediately after irradiation and appreciable stimulatory activity was retained by cells X-irradiated 14 days previously when only $0{\cdot}06\times10^5$ viable cells were present in the suspensions compared with $0{\cdot}94\times10^5$ immediately after irradiation (Table 2). Cells stored for some weeks after irradiation still retained slight stimulatory activity even when all the cells appeared to be dead as judged by dye-exclusion. Appreciable activity was found in cell preparations stored for long periods at 4° in EDTA or in serum-free medium but these preparations always contained a few dye-excluding cells. A low grade lymphocyte stimulation was obtained when cells killed by heat were used.

STIMULATION WITH ACTIVATED AUTOLOGOUS LYMPHOCYTES

Normal lymphocytes, some of which had been exposed to a pulse-stimulus of phytohaemagglutinin and some not stimulated, were incubated for several days in culture by which time a high proportion of the stimulated cells were blast-like. Both lots of cells (i.e. stimulated and unstimulated) were then X-irradiated and cultured for 5–6 days with lymphocytes obtained from a fresh sample of blood from the same donor, or from an

unrelated donor. X-irradiated unstimulated lymphocytes did not stimulate fresh cells of another donor. X-irradiated, pulse-stimulated cells produced a small but definite stimulation of short duration of 'like' cells as well as a much more marked and persistent enhancement of the stimulation obtained across the histocompatibility barrier (Table 3).

TABLE 3

ACTIVATED NORMAL LYMPHOCYTES AS STIMULANTS

Composition of culture	^{3}H-thymidine incorporation Day 2–3		Day 4–5		Day 6–7	
DH-2 (1 ml)	716		1124		1283	
DH-2 (0·5 ml) + DH-1C (0·5 ml)	597	(144)	489	(0)	353	(0)
DH-2 (0·5 ml) + DH-1S (0·5 ml)	4788	(4296)	7340	(6537)	1816	(1116)
DH-2 (0·5 ml) + NL-1C (0·5 ml)	3874	(3464)	10820	(10180)	3442	(2421)
DH-2 (0·5 ml) + NL-1S (0·5 ml)	7889	(7253)	22252	(21591)	27541	(26774)
DH-2 (0·5 ml) + EB-2 (0·5 ml)	13884		77630	(73772)	107688	(106081)
NL-2 (1 ml)	958		1898		2902	
NL-2 (0·5 ml) + DH-1C (0·5 ml)	1769	(1195)	4538	(3538)	4097	(2597)
NL-2 (0·5 ml) + DH-1S (0·5 ml)	7297	(6684)	28091	(27090)	24728	(23219)
NL-2 (0·5 ml) + NL-1C (0·5 ml)	597	(66)	1633	(606)	1523	(0)
NL-2 (0·5 ml) + NL-1S (0·5 ml)	3970	(3213)	5549	(4531)	4008	(2422)
NL-2 (0·5 ml) + EB-2 (0·5 ml)	15723		79240	(74995)	87131	(84815)
EB-2 (1 ml)	10013		6592		1931	
DH-1C (1 ml)	191		102		98	
DH-1S (1 ml)	268		105		116	
NL-1C (1 ml)	104		157		158	
NL-1S (1 ml)	557		138		251	

Cells from donor DH or NL were exposed to 20 μg of X5 PHA in 6 ml of medium for 3 days at 37°, or were incubated for the same period without stimulant (designated DH-1S, NL-1S, DH-1C and NL-1C respectively). After this time the cells were suspended in fresh medium at 2×10^6 viable cells/ml and X-irradiated with 6000 R. They were then tested against fresh cells obtained from the same donors (DN-2 and NL-2). The value shown under ^{3}H-thymidine incorporation is the mean disintegrations/min of triplicate cultures and the bracketed figure the increment of stimulation. X-irradiated EB-2 cells were used at a concentration of 2×10^5/ml.

STIMULATION WITH COMBINATIONS OF IRRADIATED LCL

Combinations of irradiated LCL cells were tested against normal lymphocytes on the theory that, if different antigens were operative, additive effects would be obtained whereas little increase in stimulation would take place, at least in certain ranges, if the same antigen, common to both cell lines, initiated the blastogenesis. It was established, in preliminary experiments, that small inocula of cells from one LCL would grow perfectly well in the presence of large numbers of irradiated cells of another LCL (Table 4).

In experiments with mixtures of normal lymphocytes and irradiated LCL mixtures the level of stimulation when two cell lines were present together was always greater than the mean of the two levels when they were present separately (Table 5). It was very difficult to be certain however that stimuli of two different types were acting. At high concentrations of LCL cells a very high level of stimulation was found even in cultures containing one LCL type and this tended to obscure any additional stimulation due to the second LCL. At low concentrations of LCL, dose effects were a factor complicating interpretation.

EFFECT OF IRRADIATED LCL CELLS ON THE LYMPHOCYTES OF OTHER SPECIES

Irradiated human LCL cells activated the lymphocytes of animals to a much lower degree than human lymphocytes. There were considerable variations with different animals—a representative selection of results is shown in Table 6. Some of the variations were related to the species of origin of the serum used in the cultures.

TABLE 4

GROWTH OF LCL CELLS (NON-IRRADIATED) IN THE PRESENCE OF IRRADIATED CELLS OF ANOTHER LCL

Non-irradiated LCL cells type No.	Irradiated LCL cells type No.	Days of culture 2	4	7
		Increment of activity*		
EB4, $0{\cdot}5 \times 10^4$	EB4, $0{\cdot}5 \times 10^6$	nil	5455	61,559
EB2, $0{\cdot}5 \times 10^4$	EB4, $0{\cdot}5 \times 10^6$	nil	4196	15,523
EB4, $0{\cdot}5 \times 10^4$	EB2, $0{\cdot}5 \times 10^6$	nil	1763	52,269
EB2, $0{\cdot}5 \times 10^4$	EB2, $0{\cdot}5 \times 10^6$	nil	5189	39,198

Disintegrations/min tritium in the acid insoluble fraction after subtraction of the value of the control cultures containing only irradiated cells. ^{3}H-thymidine was present for 24 hours before harvesting.

TABLE 5

STIMULATION OF LYMPHOCYTES BY COMBINATIONS OF IRRADIATED LCL CELLS

Irradiated LCL cells type and No. added to 10^6 blood lymphocytes	^{3}H-thymidine incorporation* Day 3	Day 4	Day 5	Day 6
EB4 ($0{\cdot}5 \times 10^5$)	6383	10,808	10,750	10,972
	(3796)	(6848)	(6504)	(7143)
EB2 ($0{\cdot}5 \times 10^5$)	40,447	87,015	144,280	150,809
	(39,155)	(85,923)	(142,638)	(148,563)
EB4+EB2 ($0{\cdot}25 \times 10^5$ of each)	30,681	85,074	102,727	120,617
	(28,742)	(82,548)	(99,736)	(117,579)
EB4 (10^5)		13,072		33,890
		(12,648)		(29,856)
EB2 (10^5)		25,085		149,742
		(22,913)		(144,606)
EB5 (10^5)		12,327		116,985
		(9144)		(111,561)
EB4+EB2 ($0{\cdot}5 \times 10^F$ of each)		22,074		117,809
		(20,438)		(113,280)
EB5+EB2 ($0{\cdot}5 \times 10^F$ of each)		20,315		158,150
		(17,657)		(152,880)
EB4 (10^5)		5376		32,803
		(4156)		(30,792)
EB2 (10^5)		28,004		148,994
		(25,964)		(145,843)
EB5 (10^5)		8771		109,157
		(7831)		(107,053)
EB4+EB2 ($0{\cdot}5 \times 10^5$ of each)		19,606		110,434
		(17,946)		(107,873)
EB4+EB5 ($0{\cdot}5 \times 10^5$ of each)		10,259		
		(8655)		
EB5+EB2 ($0{\cdot}5 \times 10^5$ of each)		15,387		143,249
		(13,880)		(140,734)

Blood lymphocytes (10^6 per mixed culture) were from normal human donors. * Calculated as for Table 3, previous paper.

A definite activation of rabbit blood lymphocytes was usually observed although this was small compared to the response of human blood lymphocytes. This level was not improved by adapting the LCL cells to rabbit serum for 2 weeks before irradiating a sample. The lymphocytes of a rabbit which had been immunized with EB4 cells were more responsive than those from a rabbit not immunized. The serum from this rabbit 'blocked' the activation of rabbit lymphocytes by irradiated EB4 cells. Antiserum to human IgG did not block the stimulation.

Pig lymphocytes responded very poorly or not at all to irradiated EB cells in cultures

TABLE 6

STIMULATION OF LYMPHOCYTES OF OTHER SPECIES

Irradiated LCL		Lymphocytes		Culture medium	^{3}H-thymidine incorporation*				
Type	No. cells	Source	No.		2 days	3 days	4 days	5 days	7 days
EB2	10^5	rabbit blood	4×10^6	Eagle's + rabbit serum	–	–	–	5941 (4745)	–
EB2	10^5	rabbit blood	2×10^6	Eagle's + rabbit serum	–	–	–	3747 (2230)	–
EB2	10^5	rabbit blood	1×10^6	Eagle's + rabbit serum	–	–	–	3101 (2415)	–
EB2	10^5	rabbit blood	2×10^6	Eagle's + rabbit serum	–	–	2402 (1172)	5012 (3764)	–
EB2	$2{\cdot}5 \times 10^5$	rabbit blood	2×10^6	Eagle's + rabbit serum	–	–	2330 (1042)	4370 (3121)	–
EB2[a]	10^5	rabbit blood	2×10^6	Eagle's + rabbit serum	–	–	2311 (nil)	2610 (890)	–
EB2[a]	$2{\cdot}5 \times 10^5$	rabbit blood	2×10^6	Eagle's + rabbit serum	–	–	3390 (250)	2248 (376)	–
EB2	10^5	pig blood	10^6	Eagle's + pig serum	–	294 (nil)	–	592 (nil)	–
EB4	10^5	pig blood	10^6	Eagle's + pig serum	–	401 (nil)	–	699 (nil)	–
EB2	10^5	pig blood	10^6	Eagle's + human serum	–	–	–	–	7320 (6211)
	10^6	pig blood	10^6	Eagle's + human serum	–	–	–	–	18,407 (17,218)
NHDL 5	10^5	pig blood	10^6	Eagle's + human serum	–	–	–	29,359 (28,440)	–
	10^6	pig blood	10^6	Eagle's + human serum	–	–	–	38,000 (37,229)	–
EB2	10^5	pig blood	10^6	Eagle's + human serum	–	–	–	–	13,999 (2619)
	10^6	pig blood	10^6	Eagle's + human serum	–	–	–	–	37,013 (25,553)
NHDL 5	10^5	pig blood	10^6	Eagle's + human serum	–	–	–	54,644 (33,224)	–
	10^6	pig blood	10^6	Eagle's + human serum	–	–	–	58,438 (33,245)	–
EB2	10^5	DBA/2 mouse spleen	2×10^6	RPMI 1640 + foetal calf serum	604 (335)	650 (511)	390 (310)	702 (360)	–
EB2	10^5	CBA mouse spleen	2×10^6	RPMI 1640 + foetal calf serum	851 (570)	510 (296)	350 (260)	602 (190)	–
EB2	10^5	rat spleen	2×10^6	Eagle's + foetal calf serum	821 (nil)	669 (nil)	962 (nil)	540 (136)	–
EB2	10^5	rat thymus	2×10^6	Eagle's + foetal calf serum	1363 (nil)	1497 (149)	2206 (1018)	1215 (678)	–
EB2	10^5	human blood	10^6	Eagle's + human serum	–	–	–	62,989	–

* Calculated as for Table 3, previous paper.

[a]: adapted to rabbit serum by culturing for 2 weeks in Eagle's medium containing 20 per cent rabbit serum.

containing pig serum but some response was obtained in cultures containing human serum. The response of the pig lymphocytes to irradiated LCL cells was no greater than the response to irradiated normal human blood lymphocytes. Mouse spleen lymphocytes were apparently not responsive to irradiated EB cells in cultures set up in Eagle's medium + foetal calf serum. This failure may have been due to the poor survival of mouse lymphocytes in this medium. In cultures set up in RPMI 1640—foetal calf serum, in which medium mouse lymphocytes survive very well, a small response was obtained which was maximal on day 2 or 3. Lymphocytes from rat thymus responded better than rat splenic lymphocytes.

DISCUSSION

It is well established from studies in many species that the intensity of the mixed lymphocyte reaction is related to the genetic disparity of the cell donors. In humans differences measured by the HL-A histocompatibility system appear to be correlated with the mixed lymphocyte response. No reaction leading to blastogenesis has been found in the absence of an antigenic difference. When only one population of lymphocytes in a mixed reaction would be expected to be capable of recognizing the foreignness of the other (as occurs in parent-F1 hybrid combinations in rats) the activated cells which develop in these cultures are confined to this population (Wilson, Silvers and Nowell, 1967). Thus 'recruitment' of cells not directly involved antigenically appears to be not a major factor.

The irradiated LCL-normal lymphocyte reaction resembles the normal mixed lymphocyte reaction in its kinetics and its requirement for living cells. It differs from it in its greater magnitude and smaller degree of variation of response between donors to the stimulus of the foreign lymphoid cells. Across a species irradiated LCL cells apparently had no special properties and were no more effective as stimulants than normal human lymphocytes. Some part of the greater effectiveness of irradiated LCL cells compared with small lymphocytes within the species in mixed cell reactions could be due simply to the larger surface area of the LCL cells. Some part could also be due to a greater quantity of antigen on the cell surface. If this were all that was involved intact 'dead' cells ought to be as good as live cells. In practice 'dead' LCL cells were effective as stimulants but much less so than viable cells. LCL cells which had been exposed to a very high dose of X-irradiation were as good as those which had been exposed to a low dose and LCL cells incubated even for as long as 7 days after X-irradiation were almost as good as freshly irradiated preparations. This might indicate that it is those irradiated LCL cells which survive for the whole culture period (almost 5–10 per cent of original preparation) which are responsible for most of the stimulation.

The general properties of LCL cells have been intensively studied during the last few years. The established cultures appear to be monoclonal judging by studies of their immunoglobulin production (Hinuma and Grace, 1967; Finegold, Fahey and Dutcher, 1968) and may have originated from a single abnormal lymphoid cell. The cell lines appear to be consistent over long periods of time in the proteins they synthesize (Fahey, Finegold, Robson and Manaker, 1966) but variations in the chromosome constitution of the Burkitt lymphoma cell lines examined at different times over a period of several years have been reported (Tough, Harnden and Epstein, 1968). The cells would be expected to express 'tumour' as well as HL-A antigens.

Experiments designed to test for 'non-antigenic metabolic' stimulation using autochthonous cells activated with a pulse of soluble mitogen did detect some presumably non-antigenic 'contagious activation' but the effect was generally small and may have been of trivial origin. In these experiments a small effect due to carry-over of mitogen cannot be entirely excluded. There is also a possible 'feeder' effect of the irradiated blast cells on the freshly isolated cells. When antigenic differences were present, however, there was no doubt that PHA-activated lymphoblasts were more effective than the same number of small lymphocytes.

We have previously presented evidence that the 'stimulating' lymphocyte in normal mixed lymphocyte reactions is something more than a carrier of HL-A or other antigens in this reaction (Hardy, Ling and Knight, 1969). Not only are X-irradiated HeLa cells and foreign red cells inactive as stimulants but X-irradiated human fibroblasts obtained from explant cultures of skin and which almost certainly contain HL-A antigens on their surfaces (Thorsby and Lie, 1968) are also inactive. Lymphocytes killed by heating to 45°, and shown by serological techniques to have retained their HL-A antigens, are likewise inactive in the mixed cell test (Schellekens and Eijsvoogel, 1970).

The most satisfactory explanation of our results is that antigenic differences, which may be small, are greatly enhanced when expressed on active lymphoid cells. The normal mixed lymphocyte reaction, to which this reaction appears to be related, may itself reflect the first stage of an immunological defence mechanism for recognition and removal of deviant lymphoid cells. A similar view relating homograft immunity to tumour immunity has gained wide acceptance (Burnet, 1969).

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