Clin. exp. Immunol. (1970) **7**, 229–239.

LYMPHOCYTE TRANSFORMATION *IN VITRO*

III. MECHANISM OF STIMULATION IN THE MIXED LYMPHOCYTE CULTURE

P. TH. A. SCHELLEKENS AND V. P. EIJSVOOGEL

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, and Laboratory of Immunopathology, University of Amsterdam

(*Received* 31 *December* 1969)

SUMMARY

In view of the correlation between the mixed lymphocyte culture and the HL-A locus, experiments were performed to determine whether the reactivity in the mixed lymphocyte culture is elicited by the HL-A antigens as such.

Fresh lymphocytes were mixed with either allogeneic fibroblasts or allogeneic lymphocytes treated with various metabolic inhibitors or by heating which abolishes their capacity to tranfsorm *in vitro*.

No stimulation of the normal untreated lymphocytes was observed in any of these mixed cultures.

However, the HL-A antigens 4b and 7b were not affected quantitatively by this treatment as determined serologically.

The conclusion is drawn that mixed lymphocyte culture reactivity is not merely a reaction to HL-A antigens, and the consequences of this are discussed.

INTRODUCTION

In a previous paper (Schellekens *et al.*, 1970) the results of family studies were described which confirm the correlation between the HL-A locus and the mixed lymphocyte culture (MLC) as reported earlier by Bach & Amos (1967).

Lymphocytes from individuals identical for the HL-A locus show no stimulation in the MLC test, regardless of incompatibilities for leucocyte antigens determined by other loci. This implies that not all leucocyte antigens are involved in the MLC.

Regarding the kinetics of transformation two major groups can be distinguished in the phenomenon of lymphocyte stimulation:

1. lymphocyte transformation reaching maximal DNA synthesis after about 72–96 hr incubation. Stimulation of this type is achieved by phytohaemagglutinin (PHA) (Nowell, 1960), streptolysin S (Hirschhorn *et al.*, 1964) and leucocyte antisera (Gräsbeck, Nordman & de la Chapelle, 1963).

Correspondence: Dr V. P. Eijsvoogel, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, P.O.B. 9190, The Netherlands.

2. lymphocyte transformation reaching maximal DNA synthesis about 144 hr after initiation of the culture. Antigen-stimulated (Pearmain, Lycette & Fitzgerald, 1963; Hirschhorn *et al.*, 1963; Ling & Husband, 1964) and mixed lymphocyte cultures (Bain, Vas & Lowenstein, 1964; Bach & Hirschhorn, 1964) belong to this category.

Quantitative differences can also be recognized: 60–80% of the cells are involved in the first type of transformation, while in general a maximum of only 40% is involved in the second. A major difference between antigen-induced stimulation and mixed lymphocyte culture is, however, that while sensitization for the particular antigen is a prerequisite for the former—this *in vitro* reaction thus representing an anamnestic response—the necessity for pre-existing immunity is not apparent for the latter. One could postulate a 'natural' background immunity for certain HL-A determined histocompatibility antigens, although no such immunity can be demonstrated by means of accelerated graft rejection.

Various investigators have tried to convert the two-way reaction of the MLC to a one-way reaction by killing the stimulating cells through repeated freezing and thawing. Some authors obtained good results (Hirschhorn *et al.*, 1963; Elves, Israëls & Booth, 1965; Iványi *et al.*, 1967), but others failed to get a reproducible one-way system (Kasakura & Lowenstein, 1965; Gordon & MacLean, 1965; Ceppellini *et al.*, 1965; Marshall, Rigo & Nielman, 1966; Main *et al.*, 1967; Ling, 1968). This might be due to antigen destruction occurring during the killing procedure in some of these experiments.

Viza *et al.* (1968) described lymphocyte stimulation induced by partly purified HL-A antigens.

It has been shown by Englefriet *et al.* (1966) that fibroblasts grown from human skin explants contain HL-A determined antigens. Supposing that the MLC is a reaction of the lymphocytes to foreign HL-A antigens as such, one would expect fibroblasts also to induce lymphocyte transformation.

Experiments bearing on these aspects of the MLC will be reported in this paper.

Lymphocytes were also treated with a number of different metabolic inhibitors to determine whether these cells

(a) could still be transformed by PHA or PPD,

(b) could act as stimulator cells to lymphocytes from unrelated individuals and thus induce a one-way MLC,

(c) still contained HL-A determined antigens 24 hr after treatment.

The period of 24 hr was chosen, as we have found that maximal sensitization in the normal MLC is achieved within that period (Schellekens & Eijsvoogel, unpublished results).

These studies provide evidence that MLC reactivity depends upon the presence of viable lymphocytes as the stimulating agent and that the stimulation is not merely a reaction to HL-A antigens as such.

MATERIALS AND METHODS

Preparation of the lymphocyte suspension

The lymphocyte suspensions were prepared as previously described (Schellekens & Eijsvoogel, 1968). All media were buffered by TRIS-HCl. The tissue culture medium used was MEM-S supplemented with 20% human AB serum, heat-inactivated for 30 min at 56°C and then filtered through 0·22 μ millipore. 100 U penicillin and 100 μg streptomycin were added per ml.

In essence the procedure was as follows:

Venous blood, with heparin as anticoagulant, was filtered through nylon wool at 37°C to remove the granulocytes. After addition of $\frac{1}{4}$ volume of 5% dextran (mean M.W. 180,000) in saline, the red cells were allowed to sediment for 30 min at 37°C.

The lymphocyte-rich supernatant plasma was removed and centrifuged, and the cells were subsequently resuspended in Earle's BSS with heparin (4 i.U. per ml), counted electronically (Coulter counter) and divided as follows:

(1) part were washed once more in Earle's BSS and resuspended in tissue culture medium,

(2) the rest were resuspended in a final concentration of $5–10 \times 10^6$ lymphocytes/ml, and either heated for 1 hr at 45°C and washed and resuspended in tissue culture medium, or incubated with one of the inhibitors mentioned below.

After addition of the inhibitors, the cells were incubated for 30 min at 37°C, washed twice and resuspended in tissue culture medium.

The inhibitors were used in the following final concentration: mitomycin-C 12·5 μg/ml, actinomycin-D 0·5 μg/ml, KCN 10^{-3} M and iodo-acetic acid (1×10^{-3} M).

The final concentration of the lymphocytes after resuspension in the tissue culture medium was always 750,000/ml.

Fibroblasts

A modification of the method described by Harnden (1960) was used.

Skin explants were fixed in chicken plasma clots and cultured in medium 199, supplemented with 20% AB serum and 20% chicken embryo extract.

When sufficient outgrowth was apparent, the cells were trypsinized and plated to grow in monolayer.

In the experiments the fibroblasts were treated in the monolayer with mitomycin-C to inhibit their own DNA-synthesis, washed twice, and tissue culture medium was added. Generally a 30 ml culture flask contained $1–1·5 \times 10^6$ fibroblasts. Cells treated in this manner remained fixed to the bottom of the flask for at least 6 days when incubated at 37°C. Alternatively the fibroblasts were trypsinized, resuspended in Earle's BSS and treated with mitomycin-C in suspension similar to lymphocytes as mentioned above. These fibroblasts were afterwards also resuspended in tissue culture medium to a final concentration of 750,000/ml.

Lymphocyte cultures

All culture tubes contained 3×10^6 lymphocytes in 4 ml of medium. All mixtures of normal lymphocytes, treated lymphocytes with normal lymphocytes, or fibroblasts with lymphocytes, were made in a ratio of 1:1. $1·5 \times 10^6$ lymphocytes were added to the fibroblast monolayers. The degree of transformation was determined by means of thymidine-2-C14 incorporation and liquid scintillation counting as previously described (Schellekens *et al.*, 1970). PHA-stimulated lymphocytes were harvested after 3 days of incubation, all other cultures after 6 days.

Serology

In the absorption experiments 0·1 ml of a two-fold dilution of an anti-4b or anti-7b serum was added to 0·05 ml of a suspension containing 40×10^6 cells/ml. The mixture was incubated for 45 min at 37°C. After centrifugation, the supernatant was tested with lymphocytes from 4b and/or 7b positive individuals, using the EDTA-agglutination technique (van Loghem *et al.*, 1958).

RESULTS

The results of the mixed lymphocyte cultures with cells, whether or not treated with inhibitors or heat, are summarized in Table 1.

After treatment with mitomycin-C the lymphocytes were still capable of stimulating allogeneic lymphocytes to transformation. Heat-treatment destroyed this capacity in all experiments performed.

TABLE 1. DNA synthesis in MLC's of lymphocytes treated in various ways (Jac = iodo-acetic acid, m = mitomycin-C, a = actinomycin-D, 45 = heated for 1 hr at 45°C) with and without additional PPD and control experiments of each cell suspension in c.p.m.

		Experiment No.							
		I		II		III		IV	
		Additional PPD							
		−	+	−	+	−	+	−	+
MLC:	A+B	1199		799		2246		1445	
	A_m+B	712		589		2102	2859	853	1509
	A+B_m	689		447		1501	2250	1088	2443
	A_m+B_m	22		50		13	49	20	49
	A_a+B			315	485	88	206	61	253
	A+B_a			286	317	34	78	92	1345
	A_a+B_a			74		0	2	20	42
	A_{Jac}+B	108				285	2354	57	1133
	A+B_{Jac}	23				322	1590	40	2141
	A_{Jac}+B_{Jac}	0				0	3	34	40
	A_{45}+B	28				17	1921	63	1137
	A+B_{45}	7				2	1935	49	1942
	A_{45}+B_{45}	10				0	0	21	22
	Donor	A	B	A	B	A	B	A	B
Controls:	PHA	4975	3487	3748	2999	5790	3950	4033	4525
	PPD			1362	2724	1995	2168	2012	2106
	PPD_m					32	22	24	25
	PPD_a			128	156	1	0	20	32
	PPD_{Jac}					8	3	28	18
	PPD_{45}					5	9	21	18
	no stimulant	9	5	87	55	74	148	143	91

In experiment III lymphocytes treated with iodo-acetic acid induced some transformation, but only 10–20% of that achieved using cells incubated with mitomycin-C. In the other two experiments inhibition was complete. Both after heat-treatment or iodo-acetic acid treatment the reactivity of the non-treated lymphocyte suspension present in the mixture appeared to be unimpaired after addition of purified protein derivative (PPD).

Incubation with actinomycin-D seems to abolish the capacity for stimulation in the MLC. However, the reactivity of the other, non-treated lymphocyte suspension to PPD is also very low, which suggests leakage of the antimetabolite from the treated to the untreated cells. For

this reason no conclusions can be drawn as to the stimulating potency of actinomycin-D-treated cells.

The results of the experiments with KCN are given in Table 2. It is evident that no effect of such treatment could be demonstrated in the MLC one-way or two-way reactions, or in control experiments with PHA or PPD. It was observed that the tissue culture medium in cultures of lymphocytes treated with KCN reached a much lower pH than usual.

KCN reacts with the cytochromeoxidase in the mitochondria (Keilin & Hartree, 1939).

TABLE 2. DNA synthesis in MLC's of lymphocytes treated with mitomycin-C (m) or KCN (CN) with and without additional PPD, and control experiments with both cell suspensions in c.p.m.

		Additional PPD	
		−	+
MLC:	A+B	485	
	A_m+B	612	
	A+B_m	379	
	A_m+B_m	40	
	A_{CN}+B	605	1653
	A+B_{CN}	609	2232
	A_{CN}+B_{CN}	531	
		Donor	
		A	B
Controls:	PHA	3583	3783
	PPD	1010	2144
	PPD_{CN}	942	2124
	no stimulant	12	7

A 50% inhibition of this enzyme system is obtained with a concentration of 10^{-8} M (Dixon & Webb, 1958). We have been using a concentration which was 10^5 times higher, and we therefore suppose that this will have been sufficient to block the respiration of the cells completely.

Nevertheless stimulation seems to be completely normal in KCN-treated cells. This suggests that the energy supply and other metabolic requirements during stimulation can be completely covered by glycolysis. As glycolysis is a far less efficient combustion of glucose, lactate acid production will proceed at a much higher ratio to fulfil the energy requirements in the presence of cyanide; this explains the pronounced drop of pH in the tissue culture medium.

Many efforts were made to stimulate lymphocytes with allogeneic fibroblasts, the fibroblasts being mixed with the lymphocytes in suspension and the cells sedimented together, or the lymphocytes put on top of a monolayer.

The results of an experiment of the former type are given in Table 3. Similar data were obtained in experiments of the latter type.

TABLE 3. DNA synthesis in MLC's treated with mitomycin-C (m) and mixed cultures of lymphocytes and fibroblasts (f) with and without additional PPD, and the control experiments in c.p.m.

		Additional PPD	
		−	+
MLC:	A+B	626	
	A_m+B	386	
	A+B_m	250	
	A_m+B_m	5	
	Af+B	11	7
	A+B_f	5	10
		Donor	
		A	B
Controls:	PHA	4326	5309
	PPD	2601	1850
	no stimulant	32	5

TABLE 4. Absorption experiments of an anti-4b serum with lymphocytes treated in various ways (Jac = iodo-acetic acid, mit. C = mitomycin-C, 45°C = heated for 1 hr at 45°C) or with fibroblasts (fibr.) and thereafter tested by the EDTA agglutination technique with leucocytes of donor I

Absorption with			^{2}Log titre*
Donor	Phenotype	Cell treatment	
—	—	—	4
I	4b+7b+	—	−
		Jac	−
		45°C	−
		fibr.	−
II	4b+7b−	—	−
		mit. C	−
		Jac	−
		45°C	−
III	4b−7b+	—	4
		mit.C	4
		Jac	4
		45°C	4

Log titre − means a negative reaction of the undiluted serum.

We have never succeeded in inducing lymphocyte transformation by the addition of fibroblasts. The control experiments, in which PPD was added to check the normal responsiveness of the lymphocytes under these conditions, were also negative. This implies some inhibitory effect of the fibroblasts on the allogeneic lymphocytes. It was therefore impossible from our experiments to decide whether or not fibroblasts are able to stimulate lymphocytes to transformation.

In Table 4 the results are given of the absorption-experiments of an anti-4b serum with

TABLE 5. Absorption experiments of an anti-7b serum with lymphocytes treated in various ways (Jac = iodo-acetic acid, 45°C = heated for 1 hr at 45°C) or with fibroblasts (fibr.) and tested afterwards in the EDTA agglutination technique with leucocytes of donor I

Absorption with			^{2}Log titre
Donor	Phenotype	Cell treatment	
—	—	—	5
I	4b+7b+	—	2
		Jac	2
		45°C	2
		fibr.	2
II	4b+7b−	—	5
		Jac	5
		45°C	5
III	4b−7b+	—	2
		Jac	2
		45°C	2
IV	4b+7b−	—	4
		Jac	5
		45°C	5

lymphocytes treated in various ways and with fibroblasts. It is evident that fibroblasts and lymphocytes treated with iodo-acetic acid, mitomycin-C or heat absorb the specific antibody as well as normal lymphocytes.

The titre of the antiserum remained unchanged after absorption with 4b-negative cells, excluding non-specific absorption.

In Table 5 the results of similar experiments with an anti-7b serum are given. These experiments show no evidence that the various ways of treating the lymphocytes affect the 4b or 7b antigen.

DISCUSSION

From the experiments described it appears that lymphocytes incubated with iodo-acetic acid or heated for 1 hr at 45°C are no longer capable of stimulating allogeneic lymphocytes to transformation.

Iodo-acetic acid shows a high affinity for sulphydryl groups. For example an irreversible inhibition of glyceraldehydephosphatedehydrogenase is achieved, and it has been shown that in the concentration used here (1×10^{-3} M) complete cessation of lymphocyte glycolysis occurs (J. A. Loos, personal communication).

Moreover, membranes of different cell-types also contain sulphydryl groups (e.g. erythrocytes, Szeinberg & Clejan, 1964), which implies that membrane activity may be sensitive to iodo-acetic acid.

Heat-treatment at 45°C is likely to destroy the thermolabile enzymes, which may be essential for cell metabolism. By means of absorption experiments we were able to demonstrate that the 4b and 7b antigens were not significantly affected by these various treatments. Unfortunately, lack of antisera of sufficient strength made it impossible to extend these absorptions to other HL-A specificities. It seems likely, however, that what was demonstrated for these two antigens will also hold good for other HL-A specificities, particularly as these glycoproteins may have a common basic structure (Davies *et al.*, 1967).

We are now faced with the fact that a number of HL-A determined antigens are still present after 24 hr on lymphocytes incubated with iodo-acetic acid or heated at 45°C, but that these cells are no longer capable of stimulating allogeneic non-treated cells.

We have never succeeded in stimulating lymphocytes with allogeneic lymphocytes killed by freezing and thawing or with extracts prepared in various ways, although some authors have reported stimulation so obtained.

However, Hardy & Ling (1969) also came to the conclusion that lymphocyte stimulation requires viable stimulator cells and that membrane antigens as such are not sufficient to induce the phenomenon.

From these data it can be postulated that the MLC may not be a reaction involving antigens alone, but is rather a reaction to the HL-A antigens together with an additional factor the production or transfer of which is inhibited by treatment with iodo-acetic acid or heating at 45°C, or a reaction to such a factor only, without participation of the antigens themselves.

In the latter case it must be assumed in view of the correlation between the MLC and the HL-A locus that the factor is now also determined by the HL-A locus.

This factor could be either secreted into and absorbed from the medium or transferred by means of direct cell to cell contact. McFarland, Heilman & Moorhead (1966) described cytoplasmic contact between lymphocytes in culture. By transfer from the stimulator cells to the responding cells this factor would then either enable the latter to react to the foreign HL-A antigen as such, or directly induce the lymphocytes to transform provided there is an HL-A incompatibility.

Actinomycin-D is known to inhibit the DNA-dependent RNA synthesis (Goldberg, Rabinowitz & Reich, 1962). Our experiments with this metabolic inhibitor were inconclusive. We could not determine whether active RNA synthesis of the stimulating cell is a necessity or whether the postulated factor is present in sufficient quantity from the beginning. Similarly, the experiments with the cell mixtures with fibroblasts were not conclusive. We have therefore not been able to demonstrate whether cell types other than lymphocytes containing HL-A antigens, are or are not able to stimulate allogeneic lymphocytes.

Recent work both *in vivo* and *in vitro* would suggest, however, that allogeneic non-lymphoid cells are unable to induce lymphocyte transformation. Although Hirschhorn (1965) and Möller, Beckmann & Lundgren (1966) described cytotoxicity by non-sensitized lymphocytes incubated on allogeneic fibroblast monolayers, a recent publication by Lund-

gren, Zukoski & Möller (1968) indicated that pure, non-sensitized lymphocytes incubated for 7 days on a monolayer of allogeneic fibroblasts do not induce cytolysis. These authors established however that polymorphonuclear leucocytes can induce damage of both autologous and allogeneic fibroblasts. It was demonstrated that lymphocyte-mediated cytolysis is dependent upon the percentage of blast-like cells in the suspension. This makes it unlikely that transformation takes place during the incubation of lymphocytes on the monolayer.

Some *in vivo* experiments also suggest that small lymphocytes cannot be stimulated by allogeneic cells containing strong transplantation antigens, unless there is a contact with allogeneic lymphocytes.

The local graft versus host assay, in which lymphocytes are injected under the kidney-capsule in allogeneic rats, is negative if the host is depleted of lymphocytes by means of lethal X-irradiation. If the lymphocyte transfer is performed in a parent–F1 combination and the parent spleen cells are inoculated under the capsule of the parental kidney which is grafted into the F1 host, a positive reaction is obtained (Elkins & Guttmann, 1968).

The F1 hybrid lymphocytes are unable to develop an immunological attack against the parental kidney or against the parental lymphocytes. Nevertheless local destruction occurs. The most likely explanation is that the parental lymphocytes are stimulated by the circulating F1 lymphocytes and that these stimulated lymphocytes cause secondary, non-specific kidney damage.

In the irradiated hamster test lymphocytes from a single donor injected into the skin of an irradiated hamster do not elicit a local inflammatory reaction. However, a reaction is found to occur when a mixture of the lymphocytes of two donors is injected or when the lymphocytes of a single donor are injected into the skin of a non-irradiated hamster which still has circulating lymphocytes (Ramseier & Streilein, 1965).

In both of the above models no reaction by the lymphocytes against the kidney or skin cells with their foreign antigens can be demonstrated; the reaction is only against the circulating lymphocytes.

Moreover, another common feature between the local graft versus host reaction and the MLC in rats is their dependence on the Ag-B locus (Elkins & Talm, 1966; Wilson, 1967).

The similarity between *in vitro* and *in vivo* reactivity suggests that non-sensitized lymphocytes outside the lymphoid organs apparently do not react to cell antigens, even when these antigens are determined by a strong histocompatibility locus.

Another possibility is that the lymphocytes we treated still contained HL-A determined antigens, but that these antigens were changed in such a way that they were no longer able to stimulate, comparable to the difference between antigenicity and immunogenicity.

Further studies will have to be performed to determine whether heat-treated or iodo-acetic acid treated cells are able to elicit humoral antibody production *in vivo*.

Cells treated as indicated above should also be tested for their ability to stimulate pre-sensitized allogeneic cells in the MLC. Experiments of this kind are currently performed in dogs. Such investigations should also provide an indication whether the MLC-test and the antigen-stimulated lymphocyte culture do differ basically or whether pre-existing immunity is of any significance in the former.

ACKNOWLEDGMENTS

We should like to express our gratitude to Prof. Dr J. J. van Loghem and Prof. Dr K. Hirschhorn for their encouraging advice and helpful suggestions.

The technical skill and dedication of Miss Birgitta Vriesendorp, the assistance of Miss M. v. d. Hart in the serological studies, and that of Dr J. A. Loos in the selection and application of the metabolic inhibitors, are gratefully acknowledged.

The leucocyte antisera were kindly provided by Prof. Dr J. J. van Rood.

REFERENCES

BACH, F. & AMOS, D.B. (1967) Hu-1: Major histocompatibility locus in man. *Science*, **156**, 1506.

BACH, F. & HIRSCHHORN, K. (1964) Lymphocyte interaction: a potential histocompatibility test *in vitro*. *Science*, **143**, 813.

BAIN, B., VAS, M.R. & LOWENSTEIN, L. (1964) The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood*, **23**, 108.

CEPPELLINI, R., FRANCESCHINI, P., MIGGIANO, V.C. & TRIDENTE, G. (1965) Directional activation of mixed lymphocyte cultures (a preliminary report). *Histoc. Test.* 225. Munksgaard, Copenhagen.

DAVIES, D.A.L., COLOMBANI, J., VIZA, D.C. & DAUSSET, J. (1967) Isoantigens of mice and their human homologues. *Histoc. Test.* 287. Munksgaard, Copenhagen.

DIXON, M. & WEBB, E.C. (1958) *Enzyme Inhibitors* VIII. *Enzymes*, p. 374. Longmans, Green, London.

ELKINS, W.L. & GUTTMANN, R.D. (1968) Pathogenesis of a local graft versus host reaction. Immunogenicity of circulating host leukocytes. *Science*, **159**, 1250.

ELKINS, W.L. & TALM, J. (1966) Identification of a single strong histocompatibility locus in the rat by normal spleen-cell transfer. *Ann. N.Y. Acad. Sci.* **129**, 573.

ELVES, M.W., ISRAËLS, M.C. & BOOTH, J. (1965) Lymphocyte transformation in cultures of mixed leucocytes. A possible test of histocompatibility. *Lancet*, **i**, 1184.

ENGELFRIET, C.P., HEERSCHE, J.N.M., EIJSVOOGEL, V.P. & V. LOGHEM, J.J. (1966) Demonstration of leucocyte iso-antigens on skin fibroblasts by means of the cytotoxic antibody test. *Vox Sang. (Basel)*, **11**, 625.

GOLDBERG, I.H., RABINOWITZ, M. & REICH, E. (1962) Basis of actinomycin action. I. DNA-binding and inhibition of RNA polymerase synthetic reactions by actinomycin. *Proc. nat. Acad. Sci. (Wash.)*, **48**, 2094.

GORDON, I. & MACLEAN, L.D. (1965) A lymphocyte-stimulating factor produced *in vitro*. *Nature (Lond.)*, **208**, 795.

GRÄSBECK, R., NORDMAN, C. & CHAPELLE, A. DE LA (1963) Mitogenic action of antileucocyte immune serum on peripheral leucocytes *in vitro*. *Lancet*, **ii**, 385.

HARDY, D.A. & LING, N. (1969) Effects of some cellular antigens on lymphocytes and the nature of the MLC reaction. *Nature (Lond.)*, **221**, 545.

HARNDEN, D.G. (1960) A human skin culture technique used for cytological examinations. *Brit. J. exp. Path.* **41**, 31.

HIRSCHHORN, K. (1965) Isoantigens and cell interactions (Ed. by J. Palm), p. 3. Wistar Institute Press Monograph, Philadelphia.

HIRSCHHORN, K., BACH, F., KOLODNY, R.L. & FIRSCHEIN, I.L. (1963) Immune response and mitosis of human peripheral blood lymphocytes *in vitro*. *Science*, **142**, 1185.

HIRSCHHORN, K., SCHREIBMAN, R.R., VERBO, S. & GRUSKIN, R.H. (1964) The action of streptolysin S on peripheral lymphocytes of normal subjects and patients with acute rheumatic fever. *Proc. nat. Acad. Sci. (Wash.)*, **52**, 1151.

IVÁNYI, D., RYCHLIKOVÁ, M., SASPORTES, M., IVÁNYI, P. & DAUSSETT, J. (1967) Leukocytes antigens and the mixed lymphocyte culture reaction. *Vox Sang. (Basel)*, **12**, 186.

KASAKURA, S. & LOWENSTEIN, L. (1965) A blastogenic factor in the medium of peripheral leukocyte cultures. *Histoc. Test.* 203. Munksgaard, Copenhagen.

KEILIN, D. & HARTREE, E.F. (1939) Cytochrome and cytochromeoxydase. *Proc. roy. Soc. B*, **127**, 167.

LING, N.R. (1968) *The Stimulation of Lymphocytes by the Products of Disrupted Cells. Lymphocyte Stimulation*, p. 124. North Holland, Amsterdam.

LING, N.R. & HUSBAND, E.M. (1964) Specific and non-specific stimulation of peripheral lymphocytes. *Lancet*, **i**, 363.

LOGHEM, J.J. v., HART, M. v.d., HIJMANS, W. & SCHUIT, H.R.E. (1958) The incidence and significance of complete and incomplete white cell antibodies with special references to the use of the C.C.T. *Vox Sang.* (*Basel*), **3**, 203.

LUNDGREN, G., ZUKOSKI, CH. F. & MÖLLER, G. (1968) Differential effects of human granulocytes and lymphocytes on human fibroblasts. *Clin. exp. Immunol.* **3**, 817.

MAIN, R.K., COLE, L.J., JONES, M.J. & HAIRE, H.M. (1967) DNA synthesis in mixed cultures of dog leukocytes: differential effect of X-radiation and freeze-thawing on cellular isoantigenicity. *J. Immunol.* **98**, 417.

MARSHALL, W.H., RIGO, S.J. & NIELMAN, S. (1966) Lymphocyte transformation and mitosis *in vitro* initiated by homologous macrophages. An improved method for histocompatibility testing. *Lancet*, **i**, 730.

MCFARLAND, W., HEILMAN, D.H. & MORHEAD, J.F. (1966) Functional anatomy of the lymphocyte in immunological reactions *in vitro*. *J. exp. Med.* **124**, 851.

MÖLLER, G., BECKMAN, V. & LUNDGREN, G. (1966) *In vitro* destruction of human fibroblasts by non-immune lymphoid cells. *Nature* (*Lond.*), **212**, 1203.

NOWELL, P.C. (1960) Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res.* **20**, 462.

PEARMAIN, G., LYCETTE, R.R. & FITZGERALD, P.H. (1963) Tuberculin-induced mitosis in peripheral blood leucocytes. *Lancet*, **i**, 637.

RAMSEIER, H. & STREILEIN, J.W. (1965) Homograft sensitivity reactions in irradiated hamsters. *Lancet*, **i**, 622.

SCHELLEKENS, P.TH.A. & EIJSVOOGEL, V.P. (1968) Lymphocyte transformation *in vitro*. I. Tissue culture conditions and quantitative measurements. *Clin. exp. Immunol.* **3**, 571.

SCHELLEKENS, P.TH.A., VRIESENDORP, BIRGITTA, EIJSVOOGEL, V.P., v. LEEUWEN, AAD., v. ROOD, J.J., MIGGIANO, V. & CEPPELLINI, R. (1970) Lymphocyte transformation *in vitro*. II. Mixed lymphocyte culture in relation to leukocyte antigens. *Clin. exp. Immunol.* **7**, 241.

SZEINBERG, A. & CLEJAN, L. (1964) Sulphydryl groups in the red cells of normal and glucose-6-phosphate dehydrogenase-deficient subjects. *Biochim. biophys. Acta* (*Amst.*), *Gen. Subjects*, **93**, 564.

VIZA, D.C., DEGANI, O., DAUSSET, J. & DAVIES, D.A.L. (1968) Lymphocyte stimulation by soluble human Hl-A transplantation antigens. *Nature* (*Lond.*), **219**, 704.

WILSON, D.B. (1967) Quantitative studies on the mixed lymphocyte interaction in rats. I. Conditions and parameters of response. *J. exp. Med.* **126**, 625.