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IN VITRO EVALUATION OF CELL-MEDIATED IMMUNITY IN MICE:

EXPERIMENTS WITH SOLUBLE AND CELLULAR ANTIGENS IN A SPLEEN–THYMUS CELL LEUCOCYTE MIGRATION TEST (LMT)

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SUMMARY

A modification of the macrophage or leucocyte migration inhibition assay usable in a murine model has been developed. The system operates with mixed spleen–thymus cell populations from mice sensitized to soluble protein or to transplantation antigens. In order to obtain significant inhibition it was found necessary to preincubate the sensitized cells with specific antigen for 24 hr prior to the migration assay. The macrophage inhibitory factor (MIF) has been found to determine the rate of migration inhibition in the mouse system whereas the role of humoral antibody is negligible. The murine MIF is not strain specific and operates in the absence of specific antigen.

Spleen–thymus cells from St/a mice sensitized to a first set C3H skin allograft were inhibited by approximately 30% while cells sensitized to a second set graft showed a 50% inhibition when migrating against C3H cells or MIF produced by confrontation between sensitized St/a lymphoid cells and C3H antigen.

INTRODUCTION

Investigations on the mechanisms operating in the Leucocyte Migration Test (LMT) have revealed that the initial interaction takes place between lymphocytes from the sensitized animal and the specific antigen (Bloom & Bennett, 1966; David, 1966; Thor *et al.*, 1968; Falk, Collste & Möller, 1969). This contact results in the release of a soluble substance which inhibits the migration *in vitro* of leucocytes or macrophages from both sensitized and non-sensitized animals. Accordingly, the substance is referred to as Migration Inhibitory Factor (MIF). The MIF has been produced by lymphoid cells from guinea-pigs, humans and rats. In its effect on migration it is strain and species unspecific (Thor *et al.*, 1968; Heise, Han & Weiser, 1968; Brostoff & Roitt, 1969; Rocklin, Meyers & David,

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1970). MIF appears to be a substance of protein nature with a molecular weight around 70,000 (Bennett & Bloom, 1968).

The present study was undertaken in order to develop a reproducible mouse LMT and to investigate the possible role of MIF in such a system.

It seemed important to have a quantitative *in vitro* assay reflecting cell-mediated immunity in mice. The mouse is widely used in transplantation research, mainly because of its genetics and the number of inbred strains available.

A capillary tube migration inhibition of an allogeneic combination of peritoneal macrophages from sensitized mice has been described by Al-Askari *et al.* (1965). Their method involves a poor cell gain making its practical use quite limited. Besides, following their protocol, we were unable to reproduce their findings. We therefore decided on mouse spleens as the source of sensitized cells for the LMT. Initially, our problem was that spleen cells migrated very nicely but that specific antigen applied directly into the LMT chambers failed to induce inhibition. When we added a small number of sensitized thymus cells, however, and preincubated for 24 hr with antigen, both soluble and cellular (particulate) antigen produced inhibition in a subsequent LMT.

MATERIALS AND METHODS

Animals

The sensitized mice were females of the highly inbred St/a strain, weighing 23–25 g. Females of a highly inbred C3H strain furnished the skin grafts. These two strains differ at the *H*-2 locus.

Immunization

(1) *Cellular antigens.* St/a mice were immunized with a first and a second set of C3H skin grafts to the back. Experiments were performed 1–2 weeks after rejection.

(2) *Soluble antigens.* St/a mice were immunized subcutaneously with six to eight injections of 25 mg bovine casein; injections were discontinued 1 week prior to the experiment.

Cell preparation

Spleen and thymus cell suspensions were washed $\times 3$ in Hanks's solution and resuspended in McCoy's tissue culture medium containing 30% foetal calf serum, penicillin and streptomycin (Gibco). In mixed suspensions (from the same pool of mice) the spleen–thymus cell ratio was 5:1. In all experiments spleens were pooled. One spleen yielded nucleated cells sufficient for ten LMT cultures.

For the preparation of purified lymphoid cell suspensions for MIF-production St/a spleen–thymus cells in McCoy's medium were incubated on cotton wool columns at 37°C for 30 min. Viability tests were performed with the eosin dye exclusion method.

Preincubation with antigen

Aliquots of 50 ml of McCoy's medium with $7{\cdot}5 \times 10^6$ nucleated St/a spleen–thymus cells per ml were incubated in 250 ml Erlenmayer flasks (in 5% CO_2 in oxygen, 37°C, 24 hr) with either (1) an equal amount of C3H spleen cells, or (2) 0·4 mg casein per ml. After incubation the suspensions were spun down at 1500 rev/min. The cell sediments were mounted

in capillary tubing for the subsequent LMT, in which the supernatant served as culture medium after being readjusted to pH 7·2 by bubbling the solution with 5% CO_2.

MIF production

Purified lymphoid cells from St/a spleen–thymus cell suspensions were incubated with specific antigen as described above ($7{\cdot}5 \times 10^6$ lymphocytes per ml McCoy's medium). After incubation the suspension was centrifuged in the cold at 5000 rev/min for 10 min. The cell sediment was discarded. The supernatant was readjusted to pH 7·2 with the 5% CO_2 gas mixture and passed through an XM-100 inert filter (Amicon, Holland) under nitrogen at 25 lb/in^2. By this procedure contaminating globulins (including whole antibody) and macroaggregates were removed. The filtrates were stored at −20°C and subsequently used as culture media in 'indirect' LMT against whole spleen cells from non-immunized animals (see below).

Leucocyte migration test (LMT)

Pooled spleen–thymus cells or spleen cells (see above) were suspended to 10% by volume in McCoy's medium and aspirated into siliconized capillary tubes with 1·4 mm internal diameter. The tubes were sealed by heating and centrifuged at 3000 rev/min for 10 min. The tubes were cut well below the cell–fluid interface in order to avoid the thrombocytes. The cell-containing blind ends were transferred without delay to the bottom of the culture chambers and fixed to the periphery with silicone wax. The chambers were Lucite rings glued to a glass plate, each plate containing eighteen 1 ml chambers. The chambers were filled with the proper medium, sealed with silicone wax and cover slips, and incubated at 37°C for 20 hr. Care was taken to obtain an equal distribution of test and control cultures over the various plates in single experiments, in order to avoid differences due to 'plate effect' (e.g. varying quality of the siliconization, insufficient cleansing, etc.). Air bubbles were avoided. After 20 hr of incubation individual areas of migration were measured by paper planimetry by means of a projection microscope. In one plate, each test culture was related to the mean control of the same plate (migration index arbitrarily 1·00).

The various control cultures were derived from pooled suspensions of sensitized cells without antigen, non-sensitized cells with and without antigen, etc.

RESULTS

Based on determination of variance in 491 LMT cultures without antigen the normal distribution shown in Fig. 1 was found. The type of distribution is Gaussian and with an arbitrarily chosen mean value of migration index of 1·00 the SD is 0·09. The 95% area thus covers migration indices from 0·82 to 1·18. Lower values represent 'inhibition', higher values 'stimulation'.

The appearance of normal and inhibited cultures is shown in Fig. 2. Our aim being to measure migration inhibition and not agglutination, membrane lysis, etc., care was taken on both test and control cultures to obtain regularly shaped, round colonies with a uniform decrease in cell density towards the periphery. This gives a well-defined but not too sharply demarcated colony, even in strongly inhibited cultures.

Fig. 3 depicts migration inhibition brought about by soluble antigen (casein). Significant inhibition of sensitized cells with specific antigen (column B) contrasted to a slight stimula-

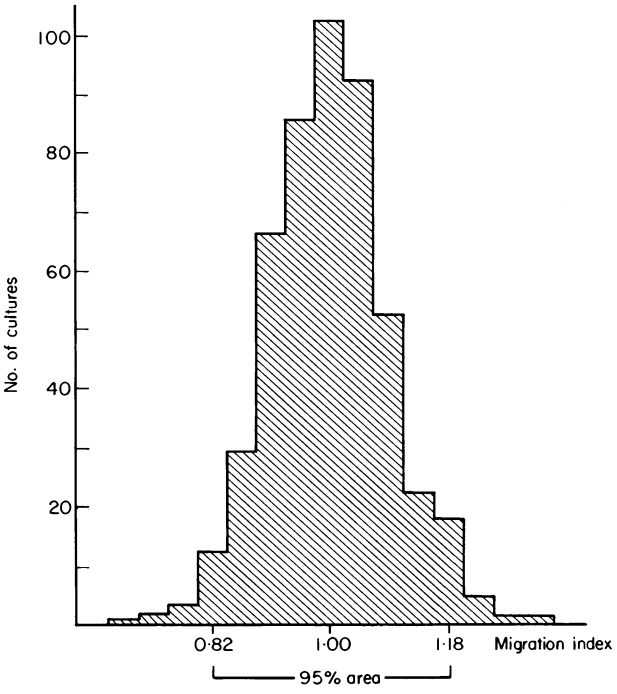

FIG. 1. Distribution of migration indices of 491 mouse spleen cell cultures migrating in McCoy's medium without antigen. In single experiments each culture is related to the mean value of the total number of cultures on a single plate (12–18).

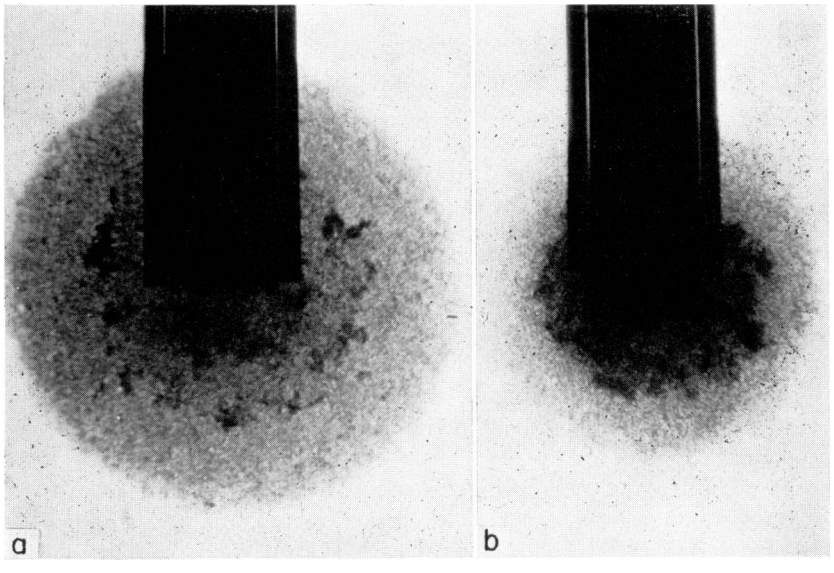

FIG. 2. (a) 20 hr control culture of whole spleen cells from normal, non-immunized St/a mice migrating in supernatant from a 24 hr incubation without antigen of lymphoid cells from St/a mice sensitized to second set C3H skin grafts. (b) 20 hr test culture of normal spleen cells from same pool as (a) migrating in supernatant from a 24 hr incubation of C3H spleen cells with lymphoid cells from St/a mice sensitized to second set C3H skin grafts (same pool as (a)). The culture has a migration index of 0·52 (inhibited 48%). Magnification × 100.

tion of non-sensitized cultures exposed to the same antigen (column E). With the antigen used, inhibition was usually evident within 5–6 days after the start of the immunization. From Fig. 3 it further appears that the degrees of inhibition by MIF produced by lymphoid cells isolated from the sensitized pool were exactly the same in cultures of migrating sensitized and non-sensitized cells (columns C and F). The same pool of MIF-containing medium

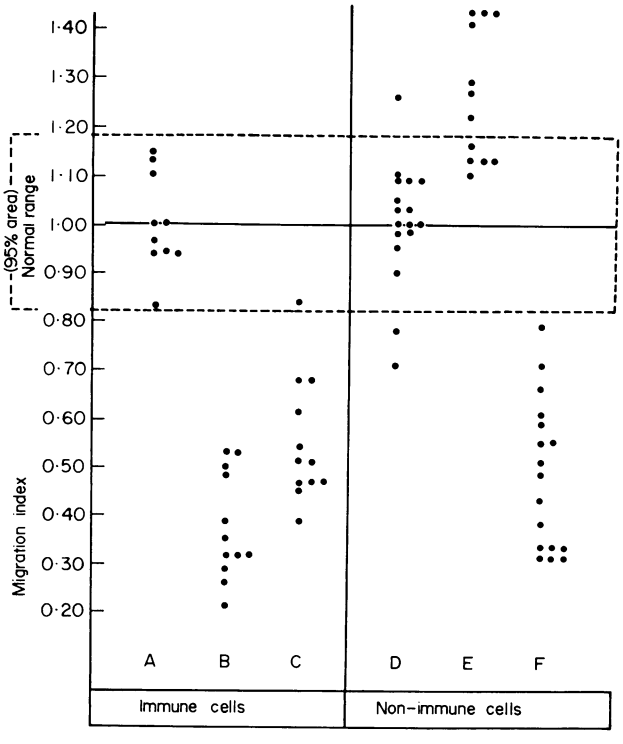

FIG. 3. A. Controls. Casein-sensitized St/a spleen-thymus cells (same pool as B and C) preincubated 24 hr without antigen. LMT: same cells, same medium.

B. Casein-sensitized St/a spleen-thymus cells (same pool as A and C) preincubated 24 hr with casein. LMT: same cells, same medium.

C. Casein-sensitized St/a spleen-thymus cells (same pool as A and B) preincubated 24 hr without antigen. LMT: same cells; medium is supernatant from a 24 hr culture of purified lymphoid cells from casein-sensitized St/a mice (same medium used in group F).

D. Controls. Non-immune St/a spleen-thymus cells (same pool as E and F) preincubated 24 hr without antigen. LMT: same cells, same medium.

E. Non-immune St/a spleen-thymus cells (same pool as D and F) preincubated 24 hr with casein. LMT: same cells, same medium.

F. Non-immune St/a spleen-thymus cells (same pool as D and E) preincubated 24 hr without antigen. LMT: same cells; same medium as used in the LMT of group C (MIF-containing).

Groups A, B, D, and E represent the 'direct LMT'; groups C and F the 'indirect LMT'.

inhibited cultures of non-sensitized spleen cells from other strains (C3H and DBA) to the same degree.

Experiments with cellular (transplantation) antigens were carried out employing first and second set C3H skin grafts to St/a mice. Inhibition was obtained invariably, being most pronounced after second set allografts. In an experiment with first set grafts a mean

migration index was found to be 0·70 (SD 0·10, $n = 18$) while LMT after a second set graft showed a mean migration index of 0·53 (SD 0·07, $n = 17$). The later experiment is depicted in Fig. 4. Control experiments with C3H-sensitized St/a cells incubated with cells derived from strains other than the immunizing one were negative, thus confirming the specificity of the reaction. When comparing the rate of migration of non-immune St/a–C3H mixtures with that of non-immune St/a alone no differences were noted, thus excluding any major cellular interaction (e.g. sensitization) *in vitro* during preincubation.

Fig. 5 illustrates the same situation as Fig. 4, now visualized in an 'indirect LMT', in which inhibition is brought about by the action of MIF being produced by lymphoid cells isolated from the same pool of C3H-sensitized St/a mice employed in the experiment pictured in Fig. 4. From Fig. 5 it is apparent that an MIF actually is produced after challenge with C3H spleen cells. The production of this MIF is antigen-specific, in that challenge with cellular antigens other than C3H cells failed to produce MIF. This is judged by the lack of inhibition of migrating spleen cells when the resulting supernatant was used as medium in an LMT. The effect of MIF, however, is independent of the strain type of migrating cells. As the various supernatants prior to use in the LMT were passed through a membrane discriminating at MW 100,000, the presence in the inhibiting (MIF-containing) media of specific antigen, whole antibody, or aggregates could be excluded. The presence in the media of Ab fragments (L chains, Fab), however, cannot be ruled out.

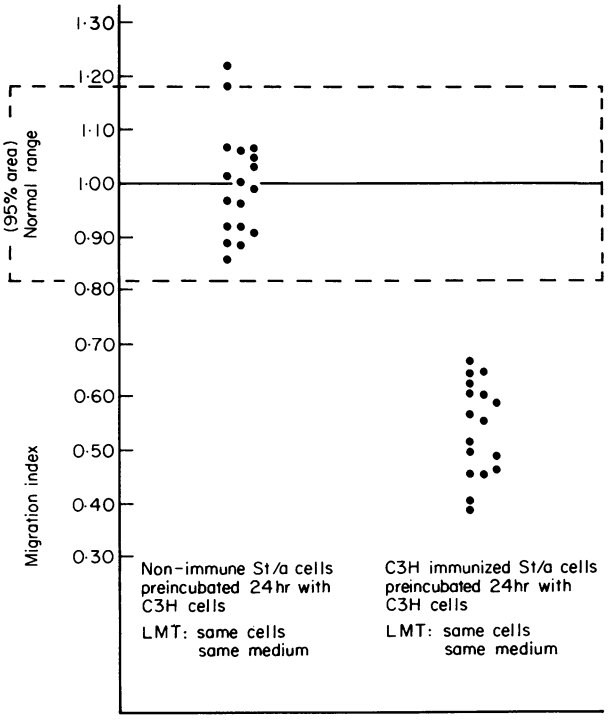

FIG. 4. Results in a 'direct LMT' experiment with spleen–thymus cells from St/a mice immunized with second set skin allografts from C3H mice. St/a cells were preincubated 24 hr with an equal amount of C3H spleen cells prior to LMT.

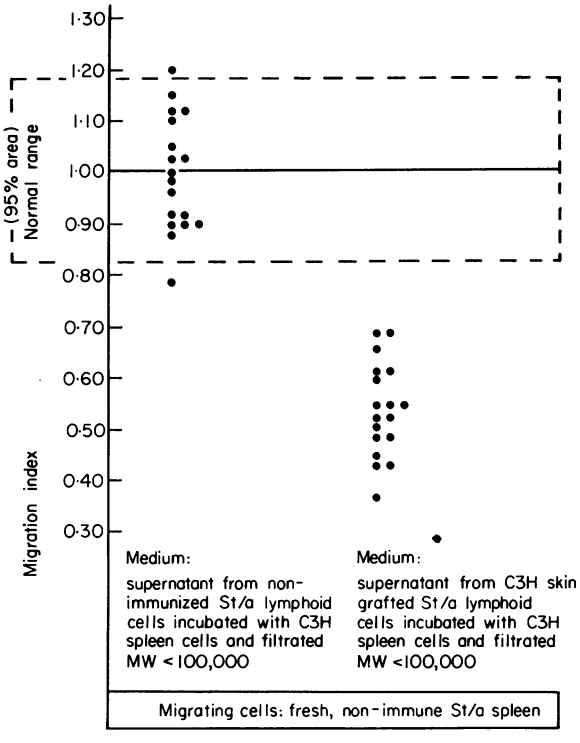

FIG. 5. Results from an 'indirect LMT' experiment with MIF-containing medium. Normal cells are inhibited by the supernatant from an incubation of immunized St/a lymphoid cells with C3H spleen cells. The St/a mice were preimmunized with second set C3H skin grafts.

The MIF of Fig. 5 causes the same degree of inhibition as the supernatant from the (pre)incubation of sensitized cells in the 'direct LMT' of the experiment depicted in Fig. 4. Both experiments drew sensitized cells from the same pool of C3H-immunized St/a cells.

DISCUSSION

The results demonstrate that the migration from capillary tubes of sensitized mouse spleen + thymus cells when cultured with specific antigen (soluble or cellular) is inhibited in a reproducible manner. Also that spleen + thymus cells from sensitized mice upon separation give rise to purified lymphoid cell suspensions which after the addition of the specific antigen produce a soluble factor capable of inhibiting migration of spleen cells derived from various mouse strains. This factor, the MIF, is present in the filtrate from a membrane discriminating at MW 100,000. When performing, the MIF seemingly does not require the presence of specific antigen. Neither the 'direct' nor the 'indirect' mouse LMT yielded false positive or false negative results.

These results extend to the murine system an acceptable means of evaluating cell-mediated immunity *in vitro*, hitherto largely restricted to the guinea-pig and the human models. Cellular immunity in the mouse has been difficult to assay because this species mostly fails to develop a positive skin test when challenged with specific antigen (Crowle, 1959). Attempts

to apply the 'blast' transformation in lymphocyte cultures as an *in vitro* test of delayed (?) hypersensitivity in mice have regularly failed, mainly because of the poor cell survival after 36–48 hr of culture (Knight *et al.*, 1965). The various migration inhibition assay systems have proved useful as *in vitro* correlates of cell-mediated immunity in guinea-pigs and there seemed to be no obvious reasons why it should not work in the mouse model too. Nevertheless, only two reports dealing with this problem have so far reached our attention. Al-Askari *et al.* (1965) adapted the peritoneal macrophage inhibition assay of George & Vaughan (1962) to allogeneic combinations of inbred mouse strains. Lately, Feinstone, Beachey & Rytel (1969) published their data from a similar model, using peritoneal exudate cells from mice sensitized with mumps or influenza virus. Both of these reports, however, disclose significant inherent difficulties in methodology and interpretation. Firstly, the collection of peritoneal macrophages in mice is a somewhat capricious procedure and the cell yield is very low, and using the common types of capillary tubes, with an internal diameter around 1 mm, one mouse seldomly yields more than one tube for LMT. For that reason, the consumption of mice per experiment must be exceedingly high if one wishes to have acceptable statistics. Next, the data of Feinstone *et al.* (1969) must have been encumbered with a number of false positives as their results included some degree of inhibition of unchallenged sensitized cells. Finally, the scarcity of cells obtained limits the possibility of MIF-production. The mouse LMT here presented seems to meet these objections: The cell gain is high, ten to fifteen cultures per animal; it works with soluble as well as cellular antigen, and antigen specificity has been established. No false positives or negatives have been encountered. MIF can be produced in sufficient amounts and produces inhibition in the absence of specific antigen.

In a migration inhibition study employing alveolar macrophages from BCG-sensitized guinea-pigs, Heise *et al.* (1968) were able to produce evidence for the presence in macrophage eluates of an IgG antibody cytophilic for macrophages. This antibody was absent in supernatants from cultures of antigen-stimulated lymphoid cells derived from sensitized guinea-pigs. Conversely, MIF was found in the latter while not in the former. The inhibiting antibody from eluates of sensitized macrophages could be passively conferred upon macrophages from normal animals. These data are consistent with the concept that 'direct' macrophage inhibition may involve cytophilic antibody as well as an inhibiting factor produced by sensitized lymphocytes (the MIF). It is not without reason to conceive that only the lymphocyte-associated factor should be regarded as reflecting the state of cellular immunity, thus stressing the importance of performing 'indirect', MIF-mediated, migration inhibition tests as proper controls in conventional LMT experiments. In the present material no attempt was made to detect humoral antibodies. However, in pilot experiments no significant differences in inhibiting property were found between ultrafiltrated and non-filtrated MIF, thus suggesting a minor role played by humoral antibody in the mouse LMT system.

It remains to be explained why the addition of sensitized thymus cells to spleen cells from sensitized mice did facilitate the inhibition by specific antigen and the production of MIF. It might be that the sustained cell-mediated immune reactivity of primed lymphocytes in part depends on a continuous provision of thymus-derived information—cell-born or otherwise. This is not incompatible with the established functional interdependence between cell-mediated immunity and the 'thymus-dependent areas' of lymphoid tissues (Parrott, DeSousa & East, 1966).

In the mouse LMT 24 hr of preincubation with the specific antigen is essential. This is not the case in experiments with guinea-pigs. This need for preincubation may be explained by a certain period of latency for MIF production by sensitized mouse lymphocytes. In a 'direct LMT' where sensitized cells are transferred directly into the capillary tubes containing specific antigen, migration may be almost at maximum before significant amounts of MIF have been formed. As migration must be a one-way phenomenon the recordings will turn out negative.

Based on the observation of inhibition of MIF-formation brought about by antilymphocytic serum the possible application of the mouse LMT as an *in vitro* assay method of ALS has already been suggested (Ranløv & Hardt, 1970).

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REFERENCES

AL-ASKARI, S., DAVID, J.R., LAWRENCE, H.S. & THOMAS, L. (1965) *In vitro* studies on homograft sensitivity. *Nature (Lond.)*, **205**, 916.

BENNETT, B. & BLOOM, B.R. (1968) Reactions *in vivo* and *in vitro* produced by a soluble substance associated with delayed-type hypersensitivity. *Proc. nat. Acad. Sci. (Wash.)*, **59**, 756.

BLOOM, B.R. & BENNETT, B. (1966) Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science*, **153**, 80.

BROSTOFF, J. & ROITT, I.M. (1969) Cell-mediated (delayed) hypersensitivity in patients with summer hayfever. *Lancet*, **ii**, 1269.

CROWLE, A.J. (1959) Delayed hypersensitivity in mice. *J. Allergy*, **30**, 151.

DAVID, J.R. (1966) Delayed hypersensitivity *in vitro*: Its mediation by cell-free substances formed by lymphoid cell–antigen interaction. *Proc. nat. Acad. Sci. (Wash.)*, **56**, 72.

FALK, R.E., COLLSTE, L. & MÖLLER, G. (1969) Release of migration inhibitory factors from immune rat lymphocytes confronted with histocompatibility antigens. *Nature (Lond.)*, **224**, 1206.

FEINSTONE, S.M., BEACHEY, E.H. & RYTEL, M.W. (1969) Induction of delayed hypersensitivity to influenza and mumps viruses in mice. *J. Immunol.* **103**, 844.

GEORGE, M. & VAUGHAN, J.H. (1962) In vitro cell migration as a model for delayed hypersensitivity. *Proc. Soc. exp. Biol. (Wash.)*, **111**, 514.

HEISE, E.R., HAN, S. & WEISER, R.S. (1968) *In vitro* studies on the mechanism of macrophage migration inhibition in tuberculin sensitivity. *J. Immunol.* **101**, 1004.

KNIGHT, S., LING, N.R., SELL, S. & OXNARD, C.E. (1965) The transformation *in vitro* of peripheral lymphocytes of some laboratory animals. *Immunology*, **9**, 565.

PARROTT, D.M.V., DE SOUSA, M.A.B. & EAST, J. (1966) Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. exp. Med.* **123**, 191.

RANLØV, P. & HARDT, F. (1970) The Migration Inhibitory Factor (MIF): Effect of ALS on formation and performance of MIF in murine *in vitro* experiments. *Transplantation* (in press).

ROCKLIN, R.E., MEYERS, O.L. & DAVID, J.R. (1970) An *in vitro* assay for cellular hypersensitivity in man. *J. Immunol.* **104**, 95.

THOR, D.E., JUREZIZ, R.E., VEACH, S.R., MILLER, E. & DRAY, S. (1968) Cell migration inhibition factor released by antigen from human peripheral lymphocytes. *Nature (Lond.)*, **219**, 755.