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Delayed Hypersensitivity Studies: some applications of cell electrophoresis

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Circulating antibody in its varying manifestations (precipitin, agglutinin, complement-fixation activity, etc.) has been studied quantitatively for many decades and its titres widely used in diagnosis. By contrast, the study of cellular (lymphocyte) sensitisation, of such importance in autoimmune disease and the immunology of transplantation, has been much hampered by the absence of a reliable and quantitative method applicable to humans (Bloom, 1971). A recently described cell electrophoresis method has these desirable features and can readily be carried out with 10 to 15 ml of blood on repeated occasions. Unfortunately, though very simple in principle, it makes heavy demands on the patience and perseverance of the operator, especially in his first months. This stems, however, from deficiencies in design of the instruments that are currently available commercially. At the moment, therefore, the technique is unlikely to become more than a research tool in clinical immunology for the study of individual cases of special interest. Some results already obtained by its use will be described, together with an indication of its scope for further studies.

Most recent studies of cellular sensitisation have used 'lymphocyte transformation'. These cells (especially human) may readily be grown in culture. If this is done in the presence of an antigen to which they are sensitised, an appreciable proportion enlarge, i.e. undergo blast-like transformation, and some go into mitosis. The degree to which an antigen induces these changes (as compared with that which might occur spontaneously in a culture or when no antigen is present) may be estimated either by counting the number of transformed cells (which is open to variation in assessment of blast cells) or more elegantly, by measuring the amount of tritiated thymidine that cultures take up as the transformed cells synthesise DNA. Unfortunately, there is considerable variation when experiments are carried out in replicate, although the exact figures are often concealed in 'averages' in published protocols. The method is useful in measuring a large response such as occurs

with phytohaemagglutinin (PHA), but is in most cases of limited value with individual antigens.

More sensitive and reproducible is the macrophage migration inhibition test introduced by David *et al.* (1964). When macrophages are put into a capillary tube that is mounted horizontally in a chamber containing a balanced salt solution and incubated at 35°C, they migrate in the space of 24 hours to form a mushroom, the area of which can be measured. If the macrophages come from an animal that has been sensitised and the antigen is present in the ambient fluid, the mushroom formed by the macrophage migration is much reduced and the reduction in area is a measure of the degree of delayed type sensitisation of the animal. It is known that inhibition of migration depends upon the interaction of antigen with the 10 to 20 per cent of lymphocytes present in the exudate to produce a protein known as macrophage migration inhibition factor (MIF) which interferes with the amoeboid movement of the macrophages. The method is widely used in animal studies; attempts to modify it for use with human leucocytes (Søborg and Bendixen, 1967) have met with varying degrees of success. Rocklin *et al.* (1970) 'encountered difficulty, as have others, in obtaining reproducible inhibition of migration by soluble antigens', though soon afterwards Rosenberg and David (1970) found the method could be made to work reasonably if special conditions (not initially specified by Bendixen) were observed. However, more recently Clausen (1971)—who had worked with Søborg on *in vitro* detection of PPD sensibility (Clausen and Søborg, 1969)—'planned to compare migration observations, using agarose plate technique parallel with the usual capillary tube method. This, however, had to be abandoned as tuberculin induced migration of leucocytes from tuberculin positive persons, tested with the capillary method, was demonstrated in only a few cases.' On the other hand, Federlin *et al.* (1971) have claimed the method to work well in their hands with PPD. This method, if it could be well standardised, should prove valuable but up to now it has not been used with consistent success. This has been our own experience.

The method presented here depends upon cell electrophoresis. This has been studied for many years (Ambrose, 1965) and it has recently been shown that the speed of migration of blood lymphocytes in an electric field is slower in the presence of antigen to which they are sensitised (Bert *et al.*, 1969). It has also been shown that the migration of peritoneal macrophages from a guinea-pig which has been immunised with a given antigen is slowed when that antigen is present (Sundarem *et al.*, 1967).

In evolving the new technique it was reasoned that, since the David test depends upon the interaction of antigen with the sensitised lymphocytes

(10 to 20 per cent) within the peritoneal macrophage exudate, it should be possible to react blood lymphocytes with antigen and perhaps produce some substance that affected electrophoretic mobility of macrophages, just as it affects their amoeboid movement. This has proved to be the case.

The principle of the test as now carried out is as follows. When lymphocytes are incubated with an antigen to which they are sensitised, an interaction takes place (involving protein synthesis; Caspary, 1971) and some material (perhaps MIF or something akin to it) is secreted into the ambient medium. This has the property of causing normal macrophages to travel more slowly in an electric field. Normal guinea-pig macrophages may thus be used as an indicator system for lymphocyte-antigen interaction. A complicating factor is the presence of 10 to 20 per cent of lymphocytes in normal guinea-pig peritoneal macrophage exudate. These lymphocytes interact with the human lymphocytes to give a 'mixed lymphocyte reaction' which itself releases macrophage slowing factor (MSF) and so interferes with the test. By exposing the exudate to 100 rads of γ irradiation from a cobalt-bomb the guinea-pig lymphocytes can be rendered incapable of taking part in a mixed reaction. Irradiation is therefore now a standard procedure in preparing the guinea-pig macrophage 'indicator system'.

Lymphocytes are isolated from venous blood (10 to 15 ml) that has been defibrinated with glass beads. The polymorphs are removed with carbonyl iron and allowed to sediment in methyl cellulose solution. About 10^6 lymphocytes are obtained from each ml of blood, 95 to 98 per cent pure, and almost 100 per cent viable (as shown by a dye exclusion test).

Normal guinea-pig macrophages are obtained by inoculating 20 ml of sterile liquid paraffin (BP) into the peritoneal cavity and washing it out 6 to 10 days later with heparinised Hanks (balanced salt) solution. After several washes to remove the heparin, the cells are suspended in medium 199. Between 60 and 200 $\times$ 10^6 cells or even many more may be obtained from one animal. Some 10 to 20 per cent of lymphocytes are present and are incapacitated, as explained above, by exposing the peritoneal exudate to 100 rads of irradiation.

In carrying out an experiment, 0·5 $\times$ 10^6 human lymphocytes to be tested are mixed with 10^7 (irradiated) macrophages and the antigen under study (usually 33 μg/ml). As a control, lymphocytes and macrophages alone, without antigen, are set up. (Usually about 18 tubes can be tested at one session.) The mixtures are incubated at room temperature (20°C) for 90 minutes and then are numbered so that a series of bijoux bottles bearing numbers 1 to 18 are presented to the observer making the readings. All measurements are thus done 'blind'.

The cell electrophoresis apparatus (Zeiss cytopherometer) consists in

essence of a chamber that can be maintained at constant temperature and is provided with two electrodes between which a steady potential difference (which is reversible) can be maintained. Cells introduced into the chamber are observed under phase contrast illumination and, by means of a grid in the microscope eyepiece, their migration under the influence of an electric field can be timed over a given distance. All measurements are limited to macrophages which are readily distinguishable by their size and liquid paraffin content. Ten macrophages are timed in each direction of the potential difference so that a mean of twenty readings may be established together with a standard deviation.

If t_c = time of migration of macrophages when lymphocytes alone are present (control time); and t_e = time when lymphocytes together with antigen are present, then, if the lymphocytes are sensitised to the antigen, $t_e > t_c$ and $(t_e - t_c/t_c) \times 100$ gives a percentage measure of the migration time increase and so of the slowing produced by the lymphocyte-antigen interaction.

The method has been compared with the macrophage migration inhibition test and found to give similar results (Hughes *et al.*, 1970). No studies involving its use have yet appeared outside our laboratory. While the method has been found to have exquisite discriminatory power in that the response of lymphocytes to a synthetic peptide which differs only minimally from the EF determinant is significantly different from that to the EF determinant itself, it suffers currently from difficulties due to inadequate design of the commercially available apparatus. This leads to intermittent drift of the macrophages even when no electric field is established and, although certain common causes are learned with practice, results in much waste of time and, indeed, in the loss of whole working days. Full exploitation of the method will wait upon the development of a stable instrument, preferably with a television display screen so that instruction of a technical assistant is facilitated.

This system can be used

(*a*) whenever the sensitisation of human blood lymphocytes to an antigen is suspected;

(*b*) to determine the antigenic reactivity of lymphocytes in disease, e.g. leukaemia, sarcoidosis, lupus erythema, collagen diseases, cancer;

(*c*) to estimate the degree of incompatibility between lymphocytes as measured by macrophage slowing in a 'mixed lymphocyte reaction' and hence for genetic studies;

(*d*) as in (*c*) as a method for tissue matching studies for transplantation work;

(*e*) possibly as a means of monitoring suppressive therapy, e.g. with anti-lymphocytic serum or cytotoxic (immunosuppressive) drugs;

(*f*) as a tool in the study of immunological processes both in health and disease.

The method would, indeed, appear to have application throughout the field of clinical immunology wherever either lymphocyte transformation or macrophage migration inhibition has been used. It gives results the same day, is exquisitely discriminatory between different antigens which differ only in a single amino acid of a short sequence (Field *et al.*, 1971) and is far more sensitive than either of the other methods mentioned. There is, for example, no difficulty in demonstrating high blood lymphocyte sensitisation to PPD in all subjects tested, while considerable inconsistencies occur with the Bendixen modification (Søborg and Bendixen, 1967) of David's technique.

Some results so far achieved are set out. All subjects examined, whether in health or disease, possess blood lymphocytes sensitised to the thyroglobulin antigen fraction of human thyroid. Only one of twenty normal subjects did not have lymphocytes sensitised to the F3 (LATS) fraction of thyroid, a finding which reflects the high sensitivity of the method, and all nineteen patients with Graves' disease showed similar sensitisation (Field *et al.*, 1970). The method can be adapted to study LATS level in blood by asking what concentration of blood serum will neutralise a given amount of LATS antigen and so prevent it from stimulating lymphocytes. In this way it has been shown that a 'high LATS' serum had approximately five times the titre of a normal subject, a result in agreement with that using Mackenzie's biological assay.

An important application of cell electrophoresis could be its use in kidney donor matching and in monitoring the development of sensitisation in the recipient of a kidney. Current methods of judging compatibility of a donor kidney with a recipient depend upon tests carried out with a bank of antigens but there is no certainty that the really important antigen is among the bank (van Rood, 1969). In using the cell electrophoresis test lymphocytes from recipient and potential donor are mixed so that a 'mixed lymphocyte response' takes place. This results in the liberation of macrophage slowing factor, which is estimated in the usual way. The question asked is 'How incompatible are the donor and recipient lymphocytes?' without reference to any particular antigen. Experiment has shown that identical twins give virtually no mixed reaction and this is the ideal situation to be aimed at. When several members of a family appear equally good potential donors by classical matching, the electrophoresis method may pick out one member as best (Table 1). It should also be possible to monitor the effect of immunosuppressive drugs by adjusting the dose until the appearance of lymphocytes incompatible with those of the donor is prevented.

TABLE 1.

(*a*) **All members of one family**

	%	P
E v. R	6·8	
– v. Y	10·1	0·01–0·005
– v. A	10·4	0·001
– v. S	10·3	0·005–0·001

E = potential recipient; R, Y, A, S = four potential donors. (Note mixed lymphocyte reaction between E and R is less than with the other three potential donors. Percentage denotes degree of macrophage slowing induced by mixed lymphocyte reaction.)
P = statistical difference from best match (E v. R)

(*b*) **Identical twins**

	%		%
Family A: $T_1 \times T_2$	1·3	*Family* B: $T_1 \times T_2$	1·0
– × Mich.	7·1	– × Ang.	6·0
– × Father	10·4	– × Father	10·2
– × Mother	6·4	– × Mother	5·2
T_2 × Mich.	7·2	T_2 × Ang.	5·5
– × Father	9·9	– × Father	10·3
– × Mother	6·9	– × Mother	5·5

T_1 and T_2 are uniovular twins in each family. Note the low values given by $T_1 \times T_2$. The twins in each case are indeed immunologically identical and this would be the ideal to aim at in the transplantation situation.

A study of the mode of action of antilymphocytic serum (ALS) has shown that it probably acts by coating the sensitised lymphocytes since no interaction occurs if ALS is presented before the antigen, but if antigen is allowed to get at the lymphocytes, the subsequent addition of ALS is without effect (Caspary *et al.*, 1970). This work offers direct experimental support for the suggestion by Levey and Medawar (1967) that ALS acts as 'blindfolding' sensitised lymphocytes and preventing their recognition of antigen.

Our main interest has been in diseases of the nervous system and, indeed, it was for further study of cellular sensitisation (delayed hypersensitivity) in multiple sclerosis that the method was developed. In 1963, E. A. Caspary first isolated from normal human brain encephalitogenic factor (EF) (Caspary and Field, 1965) capable of producing experimental allergic encephalomyelitis in 1 μg doses (with complete Freund adjuvant) in guinea-pigs. This EF is a small histone-like basic protein and the 11 amino acid sequence which goes to make up its antigenic determinant has now been worked out (Westall

et al., 1971; Carnegie *et al.*, 1971). It is to EF that sensitisation would presumably occur in multiple sclerosis (MS) if the latter were 'autoimmune' in nature. Experiment has shown that there are lymphocytes in the blood in MS which are indeed sensitised to EF, but they also occur in all other conditions in which there is destruction of brain parenchyma (Caspary and Field, 1970). Sensitisation of lymphocytes would therefore appear to be a consequence rather than a cause of nervous tissue destruction. It occurs after a week or two in head injuries with brain damage, so it could be a valuable differential aid between organic and functional nervous illness. The occurrence of sensitisation might also prove of value in assessing the physical basis of post-traumatic nervous symptoms, especially when they constitute a medico-legal problem.

The non-metastatic neurological complications of cancer (reviewed by Henson, 1970) have long presented a problem in pathogenesis, and the late Lord Brain, among others, suggested they might be immunological in origin. It was decided, therefore, to test for lymphocyte sensitisation in patients with carcinomatous cerebellar degeneration or neuropathy. This was found to be positive to central protein (EF) and to a similar basic protein material made from human sciatic nerve (SNBP), capable of producing allergic neuritis in experimental animals. As part of a control series, however, patients with carcinoma were studied who had never at any time presented symptoms or signs of nervous system involvement. It was a great surprise to find that such cases, too, showed well marked lymphocyte sensitisation both to EF and SNBP, well outside the limit found in normal subjects or those with benign growths, e.g. fibroadenoma of breast (Field and Caspary, 1970). Moreover, it was found that the magnitude of the result was unrelated to the size of growth and, indeed, carcinoma of the cervix *in situ* gave a clear positive result. Nor did surgical cure of the neoplasm result in a return to a negative finding. Even 21 years after total exenteration a patient still had a well-marked lymphocyte sensitivity. In current immunological jargon 'once a clone, always a clone' would seem to obtain. Metastasis or recurrence made no difference to the result so that the test is of no value for diagnosis here. The prolonged persistence of sensitivity may indicate that the small lymphocyte has a much longer life than the reported five years (Buckton and Pike, 1964) or that metastasis is an almost universal occurrence with small seeding growths providing a continuing immunological stimulus and themselves being held in check by lymphocytic attack. Such a state of affairs would make more understandable metastases that appear after many years of apparent cure, presumably as a result of imbalance in the equilibrium system of stimulus and check.

In the original set of results there was no overlap between controls and

cancer patients, which seemed very unusual in a biological test of this type. Above all, positive results were independent of the type of tumour. Carcinoma and sarcoma equally gave positive results. Hodgkin's disease was positive but the few cases of leukaemia tested were negative. Indeed, their lymphocytes did not even respond to PPD or F1 (thyroglobulin of thyroid).

Our results suggested that—

(*a*) it might be possible to use lymphocyte sensitisation as an early test for malignant neoplasia; and

(*b*) lymphocyte sensitisation to EF must result from changes on the surface of cancer cells or production of an EF-like antigen by neoplastic tissue.

If lymphocytes have become sensitised to some antigenic material on the surface of cancer cells, it should be possible to extract this material from tumours. This has been done and it has been found to be an even 'better' antigen in the electrophoretic test than brain EF, in the sense that it causes a greater reduction in macrophage mobility. This could mean that tumour basic protein is a 'better fit' as antigen to the sensitisation sites on lymphocytes. Moreover, it has been found that antigen made from a variety of malignant tumours (two carcinomas of breast; carcinoma of lung, stomach and vulva, as well as a lymphosarcoma) give equally good and close results, suggesting that, if the interaction of antigen with lymphocyte depends upon molecular and steric structure of the antigen, then they are all similar. Moreover, the results with tumour antigen are commonly significantly higher than with brain EF. On the other hand, in cases of organic disease of the nervous system, EF produces a higher result than does tumour basic protein (Table 2).

The two antigens must have considerable antigenic overlap but tumour basic protein is more 'fitted' to lymphocytes sensitised against malignant tissue than is brain EF, while the reverse holds true in brain disease. The difference between tumour basic protein and that extracted from normal brain (EF) may depend upon some rearrangement of amino acids in sequence or substitution (Carnegie, 1971; Field *et al.*, 1971; Caspary and Field, 1971a). By using synthetic peptides which differ only in respect of a single amino acid (either replacement or in positioning) it is possible to analyse these differences (Carnegie *et al.*, 1971; and unpublished).

The electrophoretic method of assessing lymphocyte sensitisation has many applications throughout the field of clinical immunology. As background to a study of sensitisation in cancer a survey of the population, particularly of the older age groups, needs to be done, since autoantibodies are generally found to be increased in older subjects (Walford, 1969).

Of special interest are tests carried out in patients with 'pre-malignant

TABLE 2. Selection of results obtained with EF and cancer antigens.

Patients with nervous disease

Patient	Sex	Age	EF	Ca_1	Ca_2	Ca_3	Ca_4	Ca_5	Lympho-sarc.	Diagnosis
1	F	19	20·6	14·4	14·2	14·5	—	—	—	MS
2	F	56	20·0	14·1	14·7	14·7	—	—	—	MS
3	M	68	22·9	14·0	14·8	14·5	15·1	14·5	14·3	Cerebell. deg.
4	F	38	18·0	14·1	13·7	15·0	14·1	14·5	14·9	CNS deg.

Patients with neoplasia

Patient	Sex	Age	EF	Ca_1	Ca_2	Ca_3	Ca_4	Ca_5	Lympho-sarc.	Diagnosis
1	M	67	16·2	19·6	19·1	19·9	—	—	—	Ca. bronchus
2	F	39	14·0	18·5	19·0	17·8	—	—	—	lymphosarc.
3	F	60	15·7	18·8	19·1	19·6	19·3	18·8	19·6	Ca. stomach
4	F	40	14·3	20·5	20·4	19·1	20·3	19·6	20·6	Ca. breast

Note that, in general, patients with nervous disease give higher results with EF than with neoplastic antigens, while the reverse is true in the case of patients suffering from malignant neoplasms.
EF = encephalitogenic factor from human brain; Ca_1 = epithelioma vulvae; Ca_2 = carcinoma breast 1; Ca_3 = carcinoma breast 2; Ca_4 = carcinoma stomach; Ca_5 = carcinoma bronchus.

disease' such as leukoplakia. Results are found above the normal range but below those in frank neoplasia (7 to 9 per cent as compared with 13 to 18 per cent). The same phenomenon has been observed in a family in which a very high incidence of carcinoma of the colon occurs after the age of 40, unaccompanied by polyposis coli (Dunstone and Naggs—unpublished). Where a frank cancer appears, lymphocyte sensitisation goes up to the expected level. But in those who are clinically normal, yet have a predisposition, intermediate results of the 'pre-cancerous type' have been found. Even young children (the youngest tested was two years old) show this 'pre-cancerous' sensitisation and it is clear that the condition is inherited in dominant fashion. The interpretation of such findings presents difficulties but it may be suggested that the intermediate level depends upon the occurrence of antigen of poor 'fit', rather similar to certain synthetic peptides tested which differ from true cancer antigen in a single amino acid. These, too, give intermediate values. It could be postulated that in the 'pre-cancerous state' there has been an alteration in the basic protein of normal membrane in the direction of the cancer change; a second change is needed to make the full cancer antigen.

In other words, the change from normal to cancer antigen is at least a two-step process. After the first, which may be genetically determined as in the case of the Naggs-Dunstone family, an antigen appears halfway to a cancer antigen and capable of producing an intermediate result; after the second, a true cancer antigen appears. This leaves open the causation of the second step that completes the transition from pre-cancer to cancer.

Early in the study of tumour patients, two children with Wilms' embryonic tumour were studied and (apart from sensitisation to EF) were found to have lymphocytes sensitised to PPD in as high a degree as adults. Further young children were examined and all were found to have this degree of sensitisation. Indeed, blood taken from umbilical vein at birth contained lymphocytes which gave PPD readings similar to those of the mother. These findings have been considerably extended and it appears that both in health and disease a child is born with much the same lymphocyte sensitisation as has the mother (Field and Caspary, 1971). This also offers an explanation of the fetal-maternal grafting compatibility noted by several workers.

Sarcoidosis (Caspary and Field, 1971b), Hodgkin's disease, and Sjörgren's syndrome are all conditions that would repay further study, as would the reactive capacity of lymphocytes at different stages of leukaemia (cf. Powles *et al.*, 1971). Ulcerative colitis presents many puzzling features of aetiology and pathogenesis and a few cases studied so far suggest that there is a low-grade lymphocyte sensitisation to a variety of antigens which give clear results in sarcoidosis and Crohn's disease. In particular, sensitisation to cancer antigens might be of use in studying patients who are at special risk with respect to supervening malignant change.

The presence of highly sensitised cells in cases of sarcoidosis where general anergy obtains in skin sensitivity tests suggested the existence of some lymphocyte responsiveness depressing factor in the serum. This can be readily demonstrated. Moreover, such a depressing factor is present in normal serum, though in much lower titre. Further experiment has shown the presence of lymphocyte-responsiveness depressing factor in MS, in other neurological diseases where there is parenchymatous destruction, and in cancer; indeed it appears that in all conditions where lymphocytes have become especially sensitised there develops simultaneously a responsiveness depressing factor in the serum that would tend to damp-down an over-response (Field and Caspary, 1971). The factor would appear to be a large molecule protein which is located in a single band that can readily be isolated on a Sephadex G200 column. The existence of a 'brake-accelerator' mechanism involving lymphocytes and serum factor opens up, on the one hand, the possibility of imbalance leading to disease states; and, on the other, the possible therapeutic

manipulation of the level of suppressor factor. It would be conceivable, for instance, that attacks of autoimmune disease (if such there be) might result from periodic lowering of depressing factor enabling sensitised lymphocytes to inflict tissue damage by combination with antigen in the target tissue. Under such circumstances a suppressor factor might be augmented therapeutically. On the other hand, it may be possible to lower the level of suppressor substance where lymphocyte reactivity is desirable, e.g. in an attempt to increase resistance to cancer.

In conclusion, cell electrophoresis enables lymphocyte sensitisation to be studied quantitatively in the human. At the moment the method has the drawback of requiring long and patient acquisition of expertise before reliable and reproducible results can be obtained but this situation will be remedied with the development of better apparatus. Nevertheless, it is a method of exquisite sensitivity and discrimination and should have wide applications as a research tool in clinical immunology.

References

Ambrose, E. J. (1965) *Cell Electrophoresis*. London: Churchill.
Bert, G., Di Cassono, D. L. and Pecco, P. (1969) *Clinical and Experimental Immunology*, **5**, 669.
Bloom, B. R. (1971) *New England Journal of Medicine*, **284**, 1212.
Buckton, K. E. and Pike, M. C. (1964) *Nature (Lond.)*, **202**, 714.
Carnegie, P. R. (1971) *Nature (Lond.)*, **229**, 25.
Carnegie, P. R., Caspary, E. A. and Field, E. J. (1971) *Biochemical Journal* (In the press).
Caspary, E. A. (1971) *Nature (Lond.)*, **231**, 24.
Caspary, E. A. and Field, E. J. (1965) *Annals of the New York Academy of Sciences*, **122**, 182.
Caspary, E. A. and Field, E. J. (1970) *European Neurology*, **4**, 257.
Caspary, E. A. and Field, E. J. (1971a) *British Medical Journal*, **2**, 613.
Caspary, E. A. and Field, E. J. (1971b) *British Medical Journal*, **2**, 143.
Caspary, E. A., Hughes, D. and Field, E. J. (1970) *Clinical and Experimental Immunology*, **7**, 395.
Clausen, J. E. (1971) *Acta Allergologica*, **10**, 56.
Clausen, J. E. and Søborg, M. (1969) *Acta Medica Scandinavica*, **186**, 227.
David, J. R., Al-Askari, S., Lawrence, H. S. and Thomas, L. (1964) *Journal of Immunology*, **93**, 264.
Federlin, K., Maini, R. N., Russell, A. S. and Dumonde, D. C. (1971) *Journal of Clinical Pathology*, **24**, 533.
Field, E. J. and Caspary, E. A. (1970) *Lancet*, **2**, 1337.
Field, E. J. and Caspary, E. A. (1971) *Lancet*, **2**, 337.
Field, E. J., Caspary, E. A., Hall, R. and Clark F. (1970) *Lancet*, **1**, 1144.
Field, E. J., Caspary, E. A. and Carnegie, P. R. (1971) *Nature (Lond.)*, **233**, 284.
Henson, R. A. (1970) In *Modern Trends in Neurology*, (Ed. D. Williams) p. 209. London: Butterworth.
Hughes, D., Caspary, E. A. and Field, E. J. (1970) *Zeitschrift für Immunitätsforschung und Experimentelle Therapie*, **141**, 14.
Levey, R. H. and Medawar, P. B. (1967) In *Antilymphocytic Serum*, Ciba Foundation Study Group No. 29 (Ed. G. E. W. Wolstenholme and M. O'Connor). London: Churchill.
Powles, R. L., Balchin, L. A., Fairley, H. G. and Alexander, P. (1971) *British Medical Journal*, **1**, 486.
Rocklin, R. E., Meyers, D. L. and David, J. R. (1971) *Journal of Immunology*, **104**, 95.
Rosenberg, S. A. and David J. R. (1970) *Journal of Immunology*, **105**, 1447.
Søborg, M. and Bendixen, G. (1967) *Acta medica Scandinavica*, **181**, 247.
Sundarem, K., Phondke, G. P. and Ambrose, E. J. (1967) *Immunology*, **12**, 21.
van Rood, J. J. (1969) *Lancet*, **1**, 1142.
Walford, R. L. (1969) *The Immunologic Theory of Ageing*. Copenhagen: Munksgaard.
Westall, F. C., Robinson, A. B., Caccam, J., Jackson, J. and Eylar, E. H. (1971) *Nature (Lond.)*, **229**, 22.