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IN VITRO CORRELATES OF DELAYED HYPERSENSITIVITY IN TUBERCULOSIS*

ISHWAR C. VERMA

New Delhi

It is not always easy to diagnose tuberculosis in children. Clinical examination may be unrevealing, radiology is often atypical, isolation of tubercle bacilli is more difficult, and the diagnosis is based largely on the tuberculin skin test. Introduced by von Pirquet and modified to the intradermal form by Mantoux in 1909 this test held great promise in the diagnosis of past or present infection with tuberculosis. However, the incidence of tuberculin negativity in cases of proved tuberculosis is sufficiently low in India for this test to have belied its promise. Factors responsible for this tuberculin anergy may be many. (1) Children with malnutrition have impairment of delayed dermal hypersensitivity and the degree of impairment is related to the weight of the child as a percentage of expected weight (Harland 1965) ; (2) During overwhelming tuberculous infection like miliary tuberculosis or tuberculous meningitis or even in overwhelming nontuberculous infection (Kent and Schwartz 1967) ; (3) while the patient is receiving drugs like steroids, methotrexate, nitrogen mustard or antihistaminics (Friedman 1964) ; (4) in the presence of diseases like Hodgkins' disease, sarcoidosis and hypothyroidism (Bloch 1968) ; (5) infections like paralytic poliomyelitis (Berkovich and Starr 1964) ; measles (Kipps 1966). or the administration of vaccines like measles live attenuated vaccine or oral polio vaccine (Starr and Berkovich 1964) ; and (6) lastly, sometimes the tuberculin may be deteriorated or the technique may be faulty like subcutaneous injection (Lincoln and Sewell 1963).

In view of the multiple factors which may inhibit delayed dermal hypersensitivity in tuberculosis the *in vitro* correlates of this response are receiving great attention. This work was pioneered by Chase (1945) who demonstrated that delayed dermal hypersensitivity is mediated by lymphocytes. It was expected that further understanding of this immune response will come from appropriate *in vitro* investigations of circulating lymphocytes. In 1960 Nowell discovered that phytohemagglutinin initiated mitosis in normal lymphocytes and produced blast like cells. Pearmain *et al.* (1963) and Shrek (1963) found that tuberculin had a similar effect on lymphocytes from tuberculin positive patients. This was confirmed by Cowling *et al.* (1963), Hirschhorn *et al.* (1963), and Marshall and

*From the Department of Pediatrics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-16.

Roberts (1963). Pearmain *et al.* (1963) pointed out that the ability of immunologically competent cells to respond to a specific antigen by mitosis appears to be a general character of immune response.

In 1964 Choremis *et al.* reported that leukocytes of tuberculin negative children, soon after B.C.G. vaccination, could be stimulated into mitosis by tuberculin. A considerable in vitro response was seen by the 4th day after vaccination gradually rising until the end of the 4th week after which it levelled off for the next 4 months. This response always precedes conversion of skin reaction and persists longer than the skin reaction (Matsaniotis *et al.* 1968). Elves *et al.* (1963) demonstrated that the *in vitro* lymphocyte response in the presence of P.P.D. correlated well with the development of tuberculin sensitivity after B.C.G. vaccination. This has been amply confirmed by Heilman and Mcfarland (1966), Steffani (1966), Matsaniotis *et al.* (1968) and Oppenheim (1968). These workers have pointed out that the tuberculin induced lymphocyte reactivity may be more sensitive than dermal tuberculin reactivity and that it may be useful in detecting sensitization to tuberculin not expressed by the intradermal test. Matsaniotis *et al.* (1969) found that a number of B.C.G. vaccinated children who had a negative tuberculin test showed a positive tuberculin stimulated lymphocyte reactivity and are, therefore, non-converters. They question the value of a negative tuberculin skin test in selecting candidates for B.C.G. vaccination. Oppenheim (1968) suggests that delayed hypersensitivity be redefined in terms of positive lymphocyte transformation and inhibition of macrophage migration rather than in terms of positive skin tests only.

Robbins (1964) defined lymphocyte transformation as morphological enlargement of small lymphocytes to large lymphoblasts *in vitro.* These transformed cells resemble morphologically the pyrinophilic lymphnode cells which appear after *in vivo* antigenic stimulation (Parrot and deSouza 1966). In biochemical terms there is an increased rate of histone acetylation, marked increased in protein, R.N.A. and D.N.A, synthesis and enlargement of the cells culminating in mitosis (Hirschhorn 1968). The peak of the response in terms of enlargement and cell division occurs 5 days after the onset of culture. A number of techniques are available for determining the lymphocyte transformation : (1) Enumeration of large lymphoblast like cells on stained smears of the cells show these changes although the range of the response may be from 5 to 45 per cent. This technique suffers from the drawback of confusing morphologically the blast cells with the large mononuclear cells. (2) The cultures are treated with colchicine at the end of the period of incubation thus arresting mitotic cells in metaphase stage and determining the mitotic index. Matsaniotis *et al.* (1968) found the mitotic index never to exceed 0.12% in unstimulated cultures while it was always greater than 0.4% in positively stimulated cultures, a figure three times the highest value obtained from unstimulated cultures. (3) By the addition of tritiated thymidine to the culture and subsequent autoradiography one can label the cells synthesizing D.N.A. thus unequivocally differentiattng cells which have undergone blastoid trans-

formation (Caron *et al.* 1965). (4) The most reproducible and quantitative technique for measuring the response of lymphocytes to stimulus which avoids morphological considerations altogether is an assay of the total tritiated thymidine uptake by a culture as measured by a scintillation counter (Dutton and Eady 1964).This quantitative measure of the total D.N.A. synthesis by the lymphocytes is a sensitive indicator of their transformation (Oppenheim 1968).

Other in Vitro Correlates of Delayed Hypersensitivity

In 1932, Rich and Lewis demonstrated that tuberculin inhibited the *in vitro* migration of cells taken from animals exhibiting tuberculin sensitivity. Great interest has been generated in this phenomenon by the studies of Carpenter (1963) using tissue explants and David (1964a) using the capillary tube technique of George and Vaughan (1962). This technique consists of placing peritoneal exudate cells in a thin bore capillary tube and observing their migration on to glass in lucite culture chambers. The migration of peritoneal exudate cells from guinea pigs exhibiting tuberculin sensitivity is markedly inhibited by P.P.D. while migration of cells from non-sensitized animals is not. The inhibited cells also change qualitatively and form dense clumps. This inhibition of cell migration is highly specific. The migration of cells from animals with delayed hypersensitivity to ovalbumin was inhibited by ovalbumin and not by other antigens. This inhibition could not be explained by any preformed humoral antibody present in the chambers and the specificity of the *in vitro* reaction is identical to the dermal reaction.

It has been shown that two different cell types are required for the inhibition of cell migration. The sensitive lymphocyte and the macrophage found in the peritoneal exudate (Bloom and Bennett 1966, David 1966). The latter cell has surface properties allowing it to stick to glass and to migrate in a certain manner. It is likely that this property rather than the phagocytic function makes it suitable as indicator of the *in vitro* reaction.

Early in the course of these studies it was shown that as few as 2.5% of cells from sensitive guniea pigs when mixed with cells from normal animals would cause inhibition of the whole population by antigen (David *et al.* 1964 b). These few sensitive cells had to be viable to exert their effect on mixed populations. The necessity for active cellular metabolism was further shown in studies utilizing inhibitors of protein synthesis. Sensitive cells in the presence of puromycin or its analogues that inhibit protein synthesis were no longer inhibited from migration by antigen. However, cells in the presence of analogues of puromycin that do not inhibit protein synthesis were still inhibited by antigen (David 1965).

Bloom and Bennett (1966) described experiments to ascertain the cell type responsible for the inhibition of cell migration in vitro. They separated peritoneal exudates from tuberculin sensitive guinea pigs by tissue culture methods into two cell types—macrophage populations of 99.5% homogeneity and lymphocyte populations of greater than 94% purity. It was observed that purified macrophage

populations from tuberculin sensitized guinea pigs were unaffected by the presence of antigen in the medium. In contrast when purified peritoneal lymphocytes were added to macrophages or peritoneal exudates from normal guinea pigs even in very small proportion, inhibition of migration of the normal macrophages was produced in the presence of PPD. It was concluded therefore that it is the lymphocyte which possess the immunological information, while macrophage is simply the indicator cell which migrates.

The fact that only a few lymphocytes could inhibit macrophage migration suggested that inhibition was mediated by soluble material elaborated by the lymphocyte and not by direct cell interaction. Experiments with supernatants showed that sensitised lymphocytes upon interaction with antigens elaborate macrophage migration inhibition factor. This was demonstratable as early as 6 hours after exposure of sensitized lymphocytes to antigen and was elaborated continuously for 5 days. It was felt that lymphoblastic transformation is not a prerequisite for migration inhibitory factor production as it was produced from 6 hours to 2 days when few blast cells are present. It remains to be established, however, whether cells induced to produce migration inhibitory factor proceeed to undergo transformation. Normal lymphocytes undergo more blasto-cytoid transformation when cultured in the presence of migration inhibitory factor containing supernatants as against controls. If these results are confirmed it would imply that not every transformed cell represents a sensitized cell. Interaction of a few sensitized cells with antigen may give rise to a mitogenic substance which causes lymphoblastic transformation. It is believed that sensitized cells have a specific receptor for appropriate antigen on its surface, because enzymatic treatment of sensitised cells with trypsin abolished ability of cells to produce inhibition of migration of sensitized peritoneal exudate cells in the presence of antigen. Extending this work, Bloom and Bennet (1968) found that the purified peritoneal lymphocytes or sensitized lymphnode cells after trypsinization were unable to produce the migration inhibitory factor in the presence of P.P.D. However, if after trypsinisation the cells were incubated in the presence of trypsin inhibitor they regained the ability to produce migration inhibitory factor. Pochyly (1967) showed that a similar treatment of macrophages did not impair migration inhibition. These results suggest that there may be a receptor on the surface of sensitized lymphocytes which is removed by trypsin and which can be resynthesized when trypsin activity is blocked.

The action of migration inhibitory factor is potentiated by P.P.D. and suggests that this factor may have specificity. Isolation of the migration inhibitory factor has not been successful so far but on fractionation on Sephadex G-200 column the inhibitory activity was found to be associated with the 3rd peak that contains albumin giving a molecular weight of about 70,000.

It is, however, produced by lymphocytes from only those animals with delayed type hypersensitivity. It is non-dialysable and stable to heat at 56°C for 30 minutes (David 1966). Production is blocked by Mitomycin C

and Puromycin suggesting it is probably a protein. It produces macrophage migration without killing the cells and is produced only on interaction of sensitized lymphocytes with specific antigen.

The migration inhibitory factor is yet to be implicated in production of delayed tuberculin hypersensitivity. Preliminary experiments have indicated that 10 times concentrated supernatants containing the migration inhibitory factor injected intradermally into guineapigs are capable of causing inflammatory reactions comprising pre-dominantly mononuclear cells and some neutrophils. Control supernatants have produced no or considerably smaller reactions. The relationship between these reactions and active delayed type reactions remains to be established. Thors (1968) used the capillary tube technique with human lymphnode cells in tissue culture thus providing an *in vitro* correlate of delayed hypersensitivity in humans. All donors with positive skin tests showed specific migration inhibition of their lymphnode cells. He also obtained an RNA extract which specifically and selectively confers migration inhibition on a non-sensitive cell population. This extract is sensitive to RN ase and in this respect differs from the transfer factor of Lawrence *et al.* (1963). It has a molecular weight greater than 80,000 although it might contain lower molecular weight components as the active principle. The conversion of non-sensitive cells to sensitive cells by RNA extract appears to be the result of active immunization by antigen or antigen fragments. The possibility is that RNA or RNA extract combined with an antigen fragment may provide a messenger mechanism to confer sensitivity.

In Vitro Lymphocyte Response in Active Tuberculosis

Pearmain *et al.* (1963) reported impaired blastoid transformation of lymphocytes from patients with active tuberculosis. Stefani (1966) obtained variable results. Matsaniotis *et al.* (1968) also found a positive *in vitro* lymphocyte response only in 4 out of 10 children with active tuberculosis. Lack of reactivity was thought to result from continuous exposure of lymphocytes to tuberculin which renders them incapable of responding to it by further proliferation (*vide infra*) in relation to Hodgkin's disease). Contrary to this Hirschhorn (1964) was able to obtain blast transformation of lymphocytes from tuberculous individuals. Heilman and Mcfarland (1966) offer an explanation for these conflicting results by their experiments. They suggested that there was a serum factor involved in the inhibition of rhe blastoid transformation of lymphocytes. The incubation of lymphocytes from a tuberculous patient with plasma from patients with active pulmonary tuberculosis did not lead to lymphocyte transformation in the presence of P.P.D. but their incubation in normal serum restored the response of the lymphocytes. They suggested that tuberculous serum or plasma depressed the mitogenic action of tuberculin. The inhibitory effect was not due to cytotoxic or cytolytic effect of tuberculin on sensitized lymphocytes. The inhibition was antigen specific. They argued that Hirschhorn (1968) was able to culture washed leukocytes

because he incubated them in medium containing fetal calf serum instead of autologous plasma. Matsaniotis *et al.* (1968) however observed that until more information is available the *in vitro* test during the active phase of tuberculosis must remain in doubt.

In contrast the transformation of lymphocytes from patients with Hodgkin's disease, chronic lymphocyte leukemia, Boeck's sarcoid, Sjogren's syndrome is not improved by growing them in normal serum (Oppenheim 1968) suggesting that in these diseases the lmphocyte function is due to a defect intrinsic to the cells.

Transfer of Delayed Hypersensitivity

In animals delayed hypersensitivity can be regularly transferred passively from sensitive to normal animals with cells but not with sera. In 1942 Landsteiner and Chase transferred contact sensitivity while in 1945 Chase transferred tuberculin sensitivity with viable cells from peritoneal exudates, lymphnodes and spleen. Transfer of sensitivity can be detected as early as hours after injection of cells in normal guinea pigs (Chase 1945, Metaxas and Metaxas-Buhler 1955). This sensitivity may last as long as one week and and then disappears presumably because sensitive cells may be removed in an incompatible host by homograft reaction. This transfer lasts longer in inbred animals than in random bred animals.

The exact mechanism of transfer of delayed hypersensitivity is still not known. It has been established experimentally that (1) transferred sensitive cells do not accumulate preferentially at a specific or non-specific test site as shown by injecting H^3-thymidine labelled sensitive cells. (2) Most of the cells at test site are nonsensitive recently proliferating host cells. McCluskey *et al.* (1963) demonstrated that when nonlabelled cells were injected lnto normal animals whose cells had been previously labelled by H^3-thymidine 68-91%of cells at the test site were labelled. (3) A depression of systemic sensitivity leads to depressed dermal expression of hypersensitivity. This was shown by failure of guinea pigs to show hypersensitivity when given methotrexate at the same time as antigen (Friedman *et al.* 1961). However, lymphnode suspensions from such animals were capable of transferring delayed hypersensitivity to normal donors. Methotrexate did not prevent the induction of delayed hypersensitivity but it either blocked the multiplication of sensitive cells to a sufficient number to express delayed hypersensitivity or prevented the response contributed by the nonsensitive cells at the skin test site. Similarly guinea pigs deprived of vitamin C did not express delayed hypersensitivity while cells from these animals could transfer delayed dermal hypersensitivity to normal recipients (Mueller and Kies 1962). (4) Intact metabolising cells are necessary to effect transfer in guinea pigs as incubation of sensitive cells in vitro with mitomycin inhibits tranfer of delayed hypersensitivity (Bloom *et al.* 1964). It appears therefore that a few sensitive cells could reach the site at random and after interacting with the antigen set off a reaction which involves nonsensitive cells thus leading to an expression of delayed dermal hypersensitivity.

In human beings the transfer of delayed hypersensitivity was first accomplished by Lawrence in 1945 by using peripheral leukocytes. In guinea pigs a large number of cells is required to effect transfer, as much as a lymph node or an entire peritoneal exudate. In man leukocytes from 50 to 100 ml of blood can transfer delayed hypersensitivity that may last as long as 2 years (Lawrence 1960). Strikingly in man dead cells and cell free preparations work as well as living leukocytes (Lawrence 1955, Olivera-Lima 1958) whereas in laboratory animals cellular immunity has never been transferred without living cells. It is unlikely that the transferred extract is simply boosting a mild, undetected but naturally acquired sensitivity as coccidioidomycosis sensitivity could be transferred to non-sensitive persons who had never lived near endemic areas with this fungal disease (Rapaport *et al.* 1960). This was confirmed hy Maurer (1961) by transferring delayed hypersensitivity to ethylene oxide treated human serum to subjects who were not skin tested prior to transfer, a procedure required for selection of negative recipients for transfer of bacterial or fungal proteins.

The transfer factor responsible is obtained by alternate freezing and and thawing of peripheral white blood cells from sensitized donors. After disruption of cells DNAse is added to reduce viscosity of the extract. The active material is resistant to RNAse and trypsin and is heat stable (Lawrence 1960). In 1963 Lawrence found that dialysates of sensitized leukocyte extracts were capable of transfering hypersensitivity. The dialysate has a molecular weight between 700 to 4000 (Fireman *et al.* 1967). It contains nucleic acid but is remarkably resistant to nucleases and proteases. The possibility that there may be several factors involved is suggested by the finding of Baram *et al.* (1966) that both dialysable and non-dialysable fractions of leukocytes can transfer delayed hypersensitivity in man.

Transfer of tuberculin hypersensitivity is an active process whereby information contained in the transfer factor is replicated and incorporated into effector lymphoid cells. The subject's own cells must be synthesized. Thus an intact immunological apparatus is necessary for humans to accept transfer of cellular immunity. Patients with sarcoidosis and Hodgkin's disease injected with transfer factor are still incapable of exhibiting systemic tuberculin sensitivity (Good *et al.* 1962, Mufttuolglu and Balkuv 1967). Patients with sarcoidosis have transient delayed cutaneous sensitivity when transfer factor and tuberculin are injected together. Here expression of sensitivity is purely local as the skin acts only as a passive receptacle for interaction between antigen and transfer factor (Urbach *et al.* 1962). This observation however, excludes inhibitors and other peripheral circumstances as causes for non-development of systemic cutaneous sensitivity following transfer. These results favour existence of central immunologic deficit consequent to unspecified aberration of immuno-competent cell that may either cause or result from disease. The cell population processing the immunologic information presented by the transfer factor is either mechanically displaced or functionally depressed as a result of

widespread reticulo-endothelial involvement. Alternatively the host may be so committed immunologically to antigens that the cell population concerned cannot be deflected from this pre-occupation to manufacture their own or process some one else's transfer factor.

Fireman *et al.* in 1968 were able to effect passive transfer of tuberculin reactivity *in vitro*. Leukocytes from tuberculin negative individuals cultured in the presence of a dialyzate fraction of leukocytes from tuberculin positive donor (transfer factor) with the addition of PPD resulted in leukocyte stimulation similar to that observed after addition of PPD to leukocytes from tuberculin positive individuals.

The transfer factor has also been used as a therapeutic immmune agent by Kempe (1960). A child with generalized vaccinia had shown no response to immune γ globulin. Local and systemic transfer of buffy coat and lymphnode cells resulted in prompt and dramatic regression. The patient recovered coincidentally with appearance of local but not systemic transfer of delayed hypersensitivity to killed vaccinia virus.

Summary

Since the demonstration of the lymphocyte as a mediator of delayed hypersensitivity our understanding of this immune response has advanced tremendously. It is now recognised that the lymphocyte acts as the sensitized cell and the macrophage as the indicator cell. The following mechanism for delayed hypersensitivity has been suggested. Antigen→macrophage super antigen→lymphoblast→sensitized lymphocyte→migration inhibitory factor→macrophage. The anergy to tuberculin testing in active tuberculosis and other states is better understood. It is believed that in active tuberculosis there is an inhibitory factor in the serum. The recognition of the *in vitro* correlates of delayed hypersensitivity has provided yet another method of detecting this immune response. It is more sensitive than the dermal tests. It is hoped that this will prove useful in the diagnosis and management of tuberculosis, a disease where hypersensitivity plays a large part in the pathological process.

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