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# *IN VITRO* DEMONSTRATION OF DELAYED HYPERSENSITIVITY IN PATIENTS WITH BERYLLIOSIS*

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## ABSTRACT

To clarify immunopathological mechanisms in granulomatous hypersensitivity (GHR) to beryllium (Be), migration inhibitory factor (MIF) was assayed. Blood lymphocytes from three patients with GHR to Be and two normal persons were isolated and cultured with and without BeO or other antigens. Cell-free supernatants removed daily were dialyzed, lyophilized and assayed for MIF by measuring the area of migration of normal guinea pig peritoneal exudate cells out of capillary tubes within 24 hours after exposure to the supernatant. BeO added to sensitized lymphocytes produced supernatant that decreased migration, in contrast to supernatant from non-sensitized lymphocytes, indicating that BeO-sensitized lymphocytes cultured with Be elaborate a soluble factor, MIF, which correlates with delayed hypersensitivity and may play a role in granuloma formation. It may also prove useful in diagnosis of berylliosis.

Delayed type hypersensitivity can be demonstrated in patients with berylliosis by means of skin tests *in vivo* (1) and lymphocyte transformation *in vitro* (2). The relationship of delayed hypersensitivity to induction of granuloma, however, remains unclear. To further elucidate immunopathological mechanisms in berylliosis, we assayed the induction of one mediator of cellular immunity, macrophage migration inhibitory factor (MIF), by the method of Rocklin *et al.* (3). By this method, peripheral blood lymphocytes from sensitized patients, when incubated with specific antigen *in vitro*, produce MIF which inhibits the migration of normal guinea pig peritoneal exudate cells.

## MATERIALS AND METHODS

*Subjects.* Three patients with localized, experimentally induced, granulomatous skin lesions of berylliosis and two normal subjects were studied in this experiment. Delayed hypersensitivity reactions (DHR) to beryllium salts were determined by patch testing with 0.1% beryllium fluoride (BeF) or 1.0% beryllium sulfate ($BeSO_4$), and granulomatous response was detected by intradermal injections of 10 μg–1.0 μg beryllium oxide (BeO) (4). Subjects were also skin tested for DHR to PPD (purified protein derivative of tuberculin) and SKSD (streptokinase-streptodornase).

*Lymphocyte isolation and MIF induction.* Lymphocytes from these donors were isolated by incubating plasma obtained from 50 to 100 ml blood on glass wool columns to remove adherent phagocytic cells (5). After Hanks' BSS washing, cell preparations consisting of 94% lymphocytes were distributed into quadruplicate sets of culture tubes, 1.5 ml/tube, at a concentration of $3 \times 10^6$ cells/ml TC 199 medium without serum or antibiotics (3). The tubes were incubated for 3 days at 37° C in an atmosphere of 95% air and 5% $CO_2$ with and without the test antigen BeO (1.5 μg/ml). To insure reliability of the test, PPD (10 μg/ml) and SKSD (50 units/ml) were also used as antigens for induction of MIF. The cell-free supernatants were removed each day during the period and the medium, with and without the original antigen, was replaced to the original volume in the appropriate tubes. Supernatants obtained from each tube were pooled, dialyzed in the cold against 0.15 ml NaCl and then against distilled water for a total of 48 hours, and concentrated by lyophilization. For testing, the lyophilized supernatant was made up to a 5-fold concentration of the original volume of 16 ml using TC 199 with antibiotics, 10% normal heat inactivated guinea pig serum, and 5% fetal bovine serum. Induction of MIF was performed twice in separate trials for each subject tested.

*Macrophage migration inhibition assay.* Peritoneal exudate cells (PEC) from normal guinea pigs were induced by intraperitoneal injection of light mineral oil and collected in unsiliconized glassware. The cells were washed in Hanks' BSS and suspended in TC 199 containing antibiotics. These suspensions containing macrophages and lymphocytes were drawn into capillary tubes and packed by centrifugation. The capillaries were cut at the cell-fluid interface and the cell-containing portion was placed on the bottom coverslip of Mackaness type chambers. The chambers were then covered with a second coverslip and filled with the medium to be assayed. Each sample was prepared with a minimum of four capillaries. After 24 hours' incubation at 37° C, the area of macrophage migration was projected and measured by planimetry. In calculating the experimental data, the following formula was used:

$$\% \text{ migration} = \frac{\text{area of migration in supernatant with antigen}}{\text{area of migration in supernatant without antigen}} \times 100$$

The assay was usually performed two to three times using macrophages from different guinea pigs.

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## RESULTS

The Figure shows photomicrographs of normal guinea pig macrophages which migrated out of capillaries in the presence of supernatants obtained by incubating lymphocytes from one normal person (Normal #1) and from one patient (Patient A) with test antigens BeO, PPD and SKSD. Decreased migration areas are seen whenever macrophages were incubated with a specific antigen to which the subject had positive delayed skin test reactions. Thus lymphocytes of Patient A, who had positive skin tests to all three antigens, produced MIF when stimulated by each of the three antigens. In contrast, the lymphocytes of subject $N_1$ (Normal #1), who was negative to the BeO skin test, did not produce MIF when stimulated by BeO. This finding ruled out the possibility that the concentration of BeO used in this experiment could be non-specifically cytotoxic to guinea pig macrophages. On the other hand, his lymphocytes did produce MIF only when stimulated by antigens (PPD and SKSD) which gave positive skin test reactions *in vivo*.

The quantitative results for % macrophage migration obtained by planimeter measurement are tabulated (see Table). Lymphocytes from BeO skin test positive individuals (Patients S, A, B) produced MIF which resulted in migration areas of 48%, 75% and 59% of controls.

In contrast, migration areas of 105% and 93% of controls were obtained when macrophages were incubated with supernatants from BeO stimulated lymphocytes from normal individuals ($N_1$ and $N_2$). Thus the mean migration was 58.5% ± 17.0% for the three BeO positive individuals, whereas the mean migration for the two BeO negative individuals was 97.0% ± 6.9%. This difference (by t-test) was significant at $p < 0.001$. All except Patient B were skin test positive to the other antigens studied in this experiment, PPD and SKSD. Migration area was decreased to average of 73%, 55%, 85%, 53% and 73% with PPD and of 58%, 71%, 60% and 65% with SKSD.

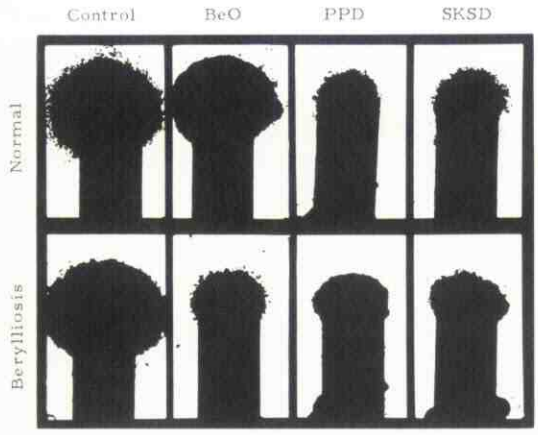


FIGURE. Macrophage migration inhibition. Compare area of migration with control. In the normal, inhibition is seen with PPD and SKSD; in the patient with berylliosis, it is seen with BeO, PPD and SKSD.

TABLE

| Subjects | BeO | | PPD | | SKSD | |
|---|---|---|---|---|---|---|
| | Patch test | % Migr. area | Skin test | % Migr. area | Skin test | % Migr. area |
| S | + | 56, 52, 31 | + | 118, 53, 49 | + | 54, 71, 51 |
| A | + | 72, 79 | + | 51, 59 | + | 68, 74 |
| B | + | 46, 73 | + | 64, 112 | | |
| $N_1$ | − | 105 | + | 53 | + | 60 |
| $N_2$ | − | 93, 93 | + | 74, 71 | + | 68, 62 |

## DISCUSSION

The results demonstrate that BeO-stimulated lymphocytes from patients with berylliosis, a metal-induced granulomatous disease, produce *in vitro* MIF, a factor which inhibits migration of normal guinea pig macrophages. The production of MIF is immunologically specific, since BeO does not stimulate lymphocytes from normal individuals to produce MIF. The induction of MIF in sensitized individuals constitutes an *in vitro* assay for delayed hypersensitivity in patients with berylliosis.

Development of *in vitro* methods has provided tools to investigate the mechanisms of delayed hypersensitivity. The original observations of George and Vaughan (6) on migration inhibition were extended by David (7), who showed that the migration of cells from guinea pigs with delayed hypersensitivity to tuberculin was consistently and markedly inhibited to physiological concentrations of PPD. Unrelated antigen had no effect on macrophage migration. When these sensitive cells were diluted with normal cells to as low as 2.5% of the total, then the entire population including the 97.5% normal cells was inhibited by antigen (8).

Bloom and Bennett demonstrated that inhibition of migration is mediated by a soluble factor, MIF, which is released by lymphocytes from sensitized donors upon contact with specific antigen (9). MIF is non-dialyzable and heat stable at 56° C for 30 minutes. MIF activity is destroyed by chymotrypsin and neuramindase but is insensitive to RNAase and DNAase (10). MIF elutes from Sephadex G 100 in a fraction after the albumin peak, its peak activity in sucrose gradient centrifugation being approximately at 35. It migrates in disc electrophoresis at pH 7.1 anodally to albumin and has a higher buoyant density than pure protein (11). These experiments suggest that MIF is an acidic glycoprotein with a molecular weight of about 40,000.

Production of MIF is inhibited by puromycin (11), suggesting that stimulated lymphocytes actively synthesize material(s) in response to antigen and do not merely release a pre-formed factor. Injection of purified preparations con-

taining MIF into the skin of normal guinea pigs provokes induration and erythema similar to a delayed skin reaction (12).

Studies by Rocklin and Thor (3, 13) showed that MIF production occurs only when the subject exhibits delayed hypersensitivity to the stimulating antigens, such as PPD, histoplasmin, coccidioidin, candida albicans and SKSD. SKSD induces MIF in almost all normal subjects tested and is considered a suitable antigen to test for anergy. The human MIF assay method has been used successfully for detection of cellular sensitivity or its lack in glomerulonephritis, sarcoidosis and Hodgkin's disease (14). The results were reproducible and correlated with *in vivo* tests for cellular hypersensitivity. The *in vitro* MIF assay is considered reliable for clinical investigation of delayed hypersensitivity (3).

Evidence suggests that berylliosis is a disease associated with delayed hypersensitivity (DHR) to beryllium (1, 15–17), similar to the findings in tuberculosis; and, as in tuberculosis, the role of DHR in the pathogenesis of granuloma formation in berylliosis remains obscure (18). Until more work is done with granulomatous diseases lacking delayed sensitivity, such as sarcoidosis and zirconium granulomas (4, 18), the present findings of MIF in berylliosis must be considered a measure of DHR only and not of granulomatous hypersensitivity.

Be exposure occurs primarily in workers engaged in beryllium processing, but rarely affects skin except by direct trauma (18). In about 50% of cases collected by the Beryllium Case Registry (BCR), the patient developed symptoms while on the job (19), but the latent period varied widely, up to 20 years from exposure to onset of clinical disease. By 1966, 535 cases of chronic berylliosis had been recorded in the BCR; the true incidence is likely much higher, since the disease frequently goes unrecognized.

Clinical diagnosis of pulmonary berylliosis can be difficult. Histologically lung sections show a diffuse interstitial non-caseating granulomatous pneumonitis (20), often indistinguishable from sarcoidosis. Patch tests with Be salts and the Kveim test have been used to differentiate berylliosis from sarcoidosis. Generally, the Kveim test is negative in berylliosis, whereas $BeSO_4$ patch tests are negative in sarcoidosis. The high incidence of false positive Kveim tests reported by Israel and Goldstein (21) in patients with persistent adenopathy of diverse causes requires confirmation before the Kveim test is abandoned. A lung biopsy for histological and metal analysis is needed to unequivocally establish a diagnosis.

The use of patch testing has several drawbacks. Despite negative results, several patients have been considered to suffer from berylliosis on the basis of history, clinical findings and lung biopsy analysis (15). Skin tests may affect the immunological status, possibly leading to contact sensitization and an altered response to inhaled Be. Exacerbations of respiratory symptoms have been reported following patch tests (16, 17). Since BeF is a potent sensitizer, patch testing would be contra-indicated for possible pre-screening for beryllium sensitivity.

*In vitro* demonstration of delayed hypersensitivity to beryllium has been reported recently. Lymphocyte transformation by BeO has been observed in patients with berylliosis (2). There is some question, however, about the specificity of lymphocyte transformation, since PHA nonspecifically induces blastogenic transformation and the correlation with delayed hypersensitivity is imperfect (22). Our *in vitro* demonstration of MIF production in patients with cutaneous berylliosis may prove a useful model in the study of pulmonary berylliosis. This assay could be used as a screening test for workers exposed to high levels of Be in the various industries where beryllium is processed and used for manufacturing purposes. A change of immunological status from a negative to a positive assay for MIF would signal a need to limit the worker's future exposure to beryllium, possibly to decrease or prevent progression to clinical disease. The test also should prove useful in screening patients suspected of having sarcoidosis.

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