Migration inhibition with various cell fractions in human colorectal cancer

P. R. ARMITSTEAD AND G. GOWLAND

From the Department of Immunology, University of Leeds, The General Infirmary, Leeds

SUMMARY The unseparated leucocytes, separated mononuclear cells and granulocytes of six control subjects and nine patients with colorectal cancer have been studied by a direct cell migration inhibition technique. A migratory index was calculated from the migration in the presence and absence of a perchloric acid extract of large bowel tumours. In 10% homologous AB serum, no significant migration inhibition occurred with any of the cells from control subjects. Five of the nine cancer patients showed significant inhibition with their unseparated leucocytes, seven of seven with their mononuclear cells, and none of nine with their granulocytes. In 10% autologous serum, some controls exhibited migration inhibition with their unseparated leucocytes and their granulocyte fraction, but not with the mononuclear cell fraction. Migration inhibition was also now apparent in the granulocyte fraction of the cancer patients. It is concluded that, with a soluble tumour antigen preparation, a mononuclear cell population increases the sensitivity of the direct migration inhibition test and that autologous serum may interfere directly with the migration of granulocytes, by an action not dependent upon the release of inhibitory factors from sensitized lymphocytes. This could explain some of the inconsistencies of the assay when using an unseparated leucocyte population.

The direct leucocyte migration inhibition technique has been extensively used in human tumour immunology and has the advantages of being rapid and relatively simple, requiring only a small amount of the patient's blood. It has the disadvantages of a lack of sensitivity and often difficult interpretation. It has been assumed to be an *in vitro* correlate of a patient's delayed hypersensitivity response to tumour associated antigens. This assumption has been questioned by Wolberg (1974), among others, who has suggested that the inhibition of leucocyte migration may be due to a chemical mediator which is synthesized by growing human tumours.

The polymorphonuclear leucocyte is often considered essential for migration inhibition, and indeed in some situations immune polymorphs alone can be inhibited from migrating without an accompanying lymphocyte population (Read and Zabriskie, 1972).

Other workers have reported that, in some situations, antibody mediates the inhibition of macrophages and leucocytes (Packalen and Wasserman, 1971). It has also been reported that the tumour patient's serum can 'block' or nullify the specific

migration inhibition obtained with tumour associated antigen preparation in homologous AB serum (Guillou and Giles, 1973).

To elucidate further the mechanism in this assay system, migration experiments were carried out in AB and autologous serum, not only with unseparated leucocyte populations but with their mononuclear and granulocyte fractions.

Methods

PATIENTS

Leucocytes were obtained from nine preoperative patients (mean age 63 years) with a diagnosis of adenocarcinoma of the large bowel. Six control subjects (mean age 63.3 years) were either healthy or had benign diseases. Their total white cell count and differential cell count were all within the normal range.

TUMOUR EXTRACT PREPARATION

A perchloric acid extract of surgically resected adenocarcinomas of the large bowel was prepared after the method of Freed and Taylor (1972). None of these tumours was autochthonous with respect

to the patients studied. The tumour extract was sterile on culture and an antiserum raised against the extract failed to agglutinate the common enteric bacteria. As used in the migration test, the extract contained 10 μ g protein per ml (Kjeldahl).

PREPARATION OF LEUCOCYTES

Unseparated leucocytes were obtained from 70 ml defibrinated venous blood by sedimentation in Dextran 150 (Fisons Ltd). Mononuclear cells and granulocytes were obtained by the Ficoll-Triosil separation of leucocytes by the method of Böyum (1974).

The three cell preparations (leucocytes, mononuclear cells, and granulocytes) were washed three times in TC 199 (Wellcome Reagents Ltd.) and resuspended to a final concentration of 20×10^6 cells per ml. The mononuclear cell fraction contained 94-96% mononuclear cells with only 4-6% granulocytes; the granulocyte fraction contained 95-97% granulocytes with 3-5% mononuclear cells. All fractions contained at least 90% viable cells as determined by a trypan blue dye exclusion test.

CELL MIGRATION TECHNIQUE

Capillary migration experiments were carried out essentially as described by Federlin (1971). Using a sterile technique, microcapillary tubes ($\frac{1}{2} \times 20 \mu$ l tubes; Microcaps, Drummond Scientific Co., U.S.A.) were two-thirds filled with unseparated or separated leucocytes and centrifuged. Tubes were cut at the cell-fluid interface and placed in disposable migration plates (Steralin) with and without antigen and in either 10% homologous AB serum or 10% autologous serum in TC 199.

After incubation at 37°C for 20 hours, migration patterns were projected at a fixed magnification, traced, and the areas measured by planimetry. The areas for each mixture, in quadruplicate, were averaged and the results expressed as a migratory index in either homologous AB serum or autologous serum.

Migratory Index =

$$\frac{\text{Area of migration with antigen}}{\text{Area of migration without antigen}}$$

Cell viability was assessed in four experiments for each cell fraction after the 20 hour culture period; in no case was the viability less than 60%.

Results

IN HOMOLOGOUS AB SERUM

Figure 1 shows the migratory indices in homologous AB serum. Normal patterns of migration were established, for each cell fraction, in AB serum with the control subject cells. Little or no migration

Fig. 1 Migratory indices of the various cell preparations from peripheral blood in the presence of the perchloric extract of colon tumours and pooled homologous AB serum. ■ Control subjects. ● Patients with carcinoma of the colon or rectum. The vertical bar represents the mean $\pm 2 \times$ SD.

inhibition was seen with the unseparated leucocytes (mean MI = 0.99 SD ± 0.66), mononuclear cell fraction (mean MI = 1.00 $\pm$ SD 1.076), or granulocyte fraction (mean MI = 0.99 $\pm$ SD 0.077). Significant inhibition of migration was therefore said to have occurred when the migratory index fell below the 95% confidence limits of these control groups (mean $-2 \times$ standard deviation).

Using this statistical method, the unseparated leucocytes from five of nine tumour patients showed significant inhibition; on the other hand the mononuclear cells from seven of seven tumour patients showed significant inhibition in the presence of the tumour extract. None of the tumour patients' granulocytes exhibited significant inhibition in AB serum.

IN AUTOLOGOUS SERUM

The effects of introducing 10% autologous serum instead of pooled homologous AB serum are shown in Fig. 2 for the control subjects. With unseparated leucocytes, migration inhibition occurred to some extent in the presence of the tumour extract; this

Fig. 2 Control subjects: a comparison of the migratory indices of various cell preparations from peripheral blood when exposed to perchloric extracts of colon tumours in the presence of either homologous AB serum or autologous serum. Vertical bars represent mean migratory index $\pm 2 \times SD$ of control group response in AB serum.

Fig. 3 Patients with carcinoma of colon or rectum: a comparison of the migratory indices of the various cell preparations from peripheral blood when exposed to a perchloric extract of colon tumour in the presence of either homologous AB serum or autologous serum. Vertical bars represent mean migratory index $\pm 2 \times SD$ of control group in AB serum.

inhibitory effect was *not* seen with their mononuclear cell fraction. However, inhibition was seen in some subjects with their granulocytes.

Figure 3 shows the effects of the tumour patients' autologous serum compared with AB serum. The

unseparated leucocytes sometimes showed an *enhancement* of the migration inhibition, while other patients exhibited an abrogation. Mononuclear cell inhibition of migration was either blocked or unaffected. No synergistic effects of autologous

serum were apparent. Autologous serum in the presence of the tumour extract tended to inhibit the migration of the tumour patients' granulocytes directly.

Discussion

In this study five of the nine tumour patients exhibited significant inhibition of migration in AB serum in the presence of the tumour extract; however, with the mononuclear cell fraction *all* the tumour patients tested showed significant inhibition. It would seem that a mononuclear cell fraction, composed predominantly of lymphocytes and monocytes can exhibit migration inhibition to our soluble tumour antigen preparation, and that the sensitivity of the assay is greater than with unseparated leucocytes. The use of a mixed human blood lymphocyte and monocyte fraction was also reported by Bull *et al.* (1973) to increase the sensitivity of the assessment of immunity of colon cancer patients by the inhibition of migration technique using a saline extract of adenocarcinoma of the colon as antigen.

However, there have been several reports that the direct LMI test using human leucocytes requires the presence of phagocytic cells together with lymphocytes for an antigen induced inhibition reaction to occur. Clausen (1970) showed that, whereas unseparated leucocytes of skin test positive persons to brucellallergen exhibited migration inhibition with brucella bacteria, the isolated granulocytes and mononuclear cell fractions did not. Similarly Read and Zabriskie (1972) using BCG as antigen, demonstrated that a mononuclear cell population alone was not inhibited in tuberculin positive patients, but that, when polymorphonuclear cells were mixed in a 50:50 ratio, the specific reactivity was regained. As these studies were with particulate antigen preparations, it may be that phagocytic cells are necessary for the processing and presentation of this type of antigen to the lymphocyte.

Senyk and Hadley (1973) found that with PPD and a streptokinase-streptodornase preparation, again a separated mononuclear cell fraction was not inhibited, but that such migration inhibition was achieved with unseparated leucocytes was reflected in a corresponding inhibition of the granulocyte fraction. They suggested that migration inhibition in their assay was due to another biologically active factor, neutrophil immobilizing factor, which was released by polymorphonuclear leucocytes. In this study, no significant migration inhibition occurred with the tumour patients' polymorphonuclear cells in AB serum in the presence of our soluble tumour

extract.

As human monocytes have been used successfully as indicator cells in a two-stage assay of cell mediated immunity to *candida albicans* (Goldberg *et al.*, 1971), it would seem probable that our soluble tumour antigen preparation directly stimulates the specifically sensitized lymphocytes to produce MIF, which then inhibits the migration of the monocytes in the mononuclear cell fraction. Rocklin's (1974) recent characterization of leucocyte inhibitory factor (LIF) by PPD, which has a different molecular weight from MIF and selectively inhibits the migration of polymorphonuclear leucocytes, cannot be incriminated in the inhibition of the mononuclear cell population. It could, however, be active in the LMI test with unseparated leucocytes. The migration inhibition in homologous AB serum observed with the tumour patients is mediated by the lymphocyte containing mononuclear cell fraction and is therefore probably a true measure of cell-mediated immunity.

Autologous serum in the presence of the tumour extract had an unexpected inhibitory effect on leucocyte migration in some control subjects. This effect was not apparent with their mononuclear cells but appeared to be due to an inhibition of the granulocyte fraction. As the results are expressed as a migratory index, this inhibitory action cannot be due solely to a component of the autologous serum; furthermore, the tumour extract did not have an inhibitory effect upon the same cells in AB serum. The likely explanation is an interaction of a component of the tumour extract with an autologous serum factor.

A similar effect of autologous serum and tumour extract was seen with the tumour patient's granulocytes. The serum factor, whatever its nature, is obviously not tumour specific, although it is of interest that the two subjects who exhibited the greatest granulocyte inhibitory effect had benign diseases of the large bowel (diverticulitis and fissure-in-ano).

That antigen-antibody complexes are able to inhibit macrophage mobility in the macrophage migration system was first reported by Carpenter (1963). This was confirmed by Bloom and Bennett (1966) who found that sera of immune animals, containing circulating antibodies, inhibited the migration of normal peritoneal exudate cells when specific antigen was present. In the human LMI test, Brostoff *et al.* (1973) showed migration inhibition to aggregated IgG. Of a group of 22 patients with rheumatoid arthritis, IgG antiglobulins were detected in 12; in these patients a good correlation was obtained between the extent of the leucocyte migration inhibition and the antibody titre. They suggested a cross-linking of the leucocytes through

immune complex formation between aggregated IgG and cytophilic antiglobulins as the mechanism. The inhibition of the granulocyte migration, in the absence of lymphocytes, seen in this study, could similarly be explained by the formation of inhibitory complexes between an autologous serum antibody and a normal tissue antigen in the tumour extract.

The effect of some tumour patients' autologous serum in abrogating the cell-mediated migration inhibition seen with the tumour extract in AB serum suggests that there is a serum blocking factor in a proportion of tumour patients' sera. This factor could be analogous to that seen in human lymphocyte cytotoxicity assay techniques (Hellstrom *et al.*, 1971) and also described by Halliday (1971) in animals with growing tumours and would provide an 'escape' mechanism from any tumour immune response in the host.

Thus, it is a compounding of the direct inhibition of granulocyte migration and the blocking effect of autologous serum on specific mononuclear cell migration inhibition that is observed with unseparated leucocyte populations, as normally used in the LMI technique. The absence of a serum blocking effect would allow the predominance of the granulocyte migratory inhibitory effect and an apparent enhancement of leucocyte migration inhibition in autologous serum. Lacour *et al.* (1974) recently reported a similar enhancing effect of autologous serum on migration inhibition in breast carcinoma and found that it correlated with the best histological prognostic factors. They suggested that the interaction of leucocytes, tumour extract, and autologous serum in this assay serum may be a reflection of the *in vivo* host's defences of a particular patient to his own tumour.

It would seem, therefore, that with a soluble tumour antigen preparation the mononuclear cell fraction not only increases the sensitivity of *in vitro* cell migration techniques, but also can assay the blocking activity of the tumour patient's serum.

We thank the surgeons of Leeds (St. James's) University Hospital for allowing us to study patients under their care.

References

- Bloom, B. R., and Bennett, B. (1966). Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science*, **153**, 80-82.
- Böyum, A. (1974). Separation of blood leucocytes, granulocytes and lymphocytes. *Tissue Antigens*, **4**, 269-274.
- Brostoff, J., Howell, A., and Roitt, I. M. (1973). Leucocyte migration inhibition with aggregated gamma globulin in patients with rheumatoid arthritis. *Clinical and Experimental Immunology*, **15**, 1-7.
- Bull, D. M., Leibach, J. R., Williams, M. A., and Helms, R. A. (1973). Immunity to colon cancer assessed by antigen-induced inhibition of mixed mononuclear cell migration. *Science*, **181**, 957-959.
- Carpenter, R. C. (1963). *In vitro* studies of cellular hypersensitivity. 1. Specific inhibition of migration of cells from adjuvant-immunized animals by purified protein derivative and other protein antigens. *Journal of Immunology*, **91**, 803-818.
- Clausen, J. E. (1970). Polymorphonuclear leucocytes in the specific antigen-induced inhibition of the *in vitro* migration of human peripheral leucocytes. *Acta Medica Scandinavica*, **188**, 59-64.
- Federlin, K., Main, R. N., Russell, A. S., and Dumonde, D. C. (1971). A micromethod for peripheral leucocyte migration in tuberculin sensitivity. *Journal of Clinical Pathology*, **24**, 533-536.
- Freed, D. L. J., and Taylor, G. (1972). Carcino embryonic antigen in faeces. *British Medical Journal*, **1**, 85-87.
- Goldberg, L. S., Louie, J. S., and Baker, M. H. (1971). Inhibition of macrophage migration. A test system using human monocytes. *Journal of Immunology*, **107**, 906-909.
- Guillou, P. J., and Giles, G. R. (1973). Inhibition of leucocyte migration by tumour-associated antigens of the colon and rectum. *Gut*, **14**, 733-738.
- Halliday, W. J. (1971). Blocking effect of serum from tumour-bearing animals on macrophage migration inhibition with tumour antigens. *Journal of Immunology*, **106**, 855-857.
- Hellström, I., Sjögren, H. O., Warner, G., and Hellström, K. E. (1971). Blocking of cell-mediated tumor immunity by sera from patients with growing neoplasms. *International Journal of Cancer*, **7**, 226-237.
- Lacour, F., Lacour, J., Spira, A., and Bayet, S. (1974). Effect of autologous serum on *in vitro* inhibition of leucocyte migration by autochthonous tumor extracts from human patients. In *Recent Results in Cancer Research*, vol. 4, pp. 129-132. Edited by G. Mathé and R. Weiner. Springer: Berlin.
- Packalén, T., and Wasserman, J. (1971). Inhibition of migration of normal guinea pig blood leukocytes by homologous immune γ_2 globulin in presence of specific antigen. *International Archives of Allergy and Applied Immunology*, **41**, 790-796.
- Read, S. E., and Zabriskie, J. B. (1972). Cellular interactions in the leucocyte migration inhibition system. *Transplant Proceedings*, **4**, 247-252.
- Rocklin, R. E. (1974). Products of activated lymphocytes: Leucocyte inhibitory factor (L.I.F.) as distinct from migration inhibitory factor (MIF). *Journal of Immunology*, **112**, 1461-1466.
- Senyk, G., and Hadley, W. K. (1973). *In vitro* correlates of delayed hypersensitivity in man: ambiguity of polymorphonuclear neutrophils as indicator cells in leucocyte migration test. *Infectious Immunology*, **8**, 370-380.
- Wolberg, W. (1974). Inhibition of leucocyte migration by human tumours. *Archives of Surgery*, **109**, 211-214.