

Demonstration of a Complement-Dependent Migration Inhibitory Activity in Normal Guinea Pig Serum

G. SANDRU and P. VERAGUTH

Laboratory of Radiobiology, Department of Radiotherapy, University of Berne, Inselspital, Berne, Switzerland

A cell migration inhibitory effect was evidenced in normal guinea pig serum as compared with heat inactivated serum. Granulocytes when used as target showed a greater sensitivity to this effect than lymphomonocytes or macrophages. The migration inhibitory activity of GPS is abrogated or decreased by using complement destroying agents such as: heating at 56°C for as little as 5 min, absorption on immune complexes in presence of calcium, on zymosan, on Sephadex G-50 or by adding EDTA or heparin to culture medium. The GPS dialysation fractions while exhibiting neither complement haemolytic effect nor migration inhibitory activity when tested alone, restored these functions by recombination. Absorption of GPS on homologous blood cells abrogated the migration inhibitory effect but retained the complement haemolytic function. When GPS absorbed on homologous blood cells was mixed 1:5 with heat-inactivated serum (5 min at 56°C), the migration inhibitory activity was regained, suggesting that the complement factors from the first sample were necessary for manifestation of the migration inhibitory activity from the heat-inactivated serum.

Key words:

Accepted for publication 29 June 1979

Different agents, including lymphokines (1-4), antigen-antibody complexes (5), cytophilic antibodies (6, 7), polymorphonuclear leucocyte products (8), or components of the coagulation system (9, 10), have been shown to inhibit cell migration *in vitro*.

MIF is reported to be produced not only by activated lymphocytes following specific or non-specific stimulation, but also by cells of established lymphoid or non-lymphoid origin (11, 12). Recently, MIF has been found in fresh non-inactivated foetal calf serum and it was claimed to be very similar to that produced by antigen-stimulated lymphocytes (13).

During MIF assay experiments in guinea pigs, we have observed that non-inactivated

autologous or homologous serum exhibited a significant inhibitory effect upon cell migration *in vitro* compared with a similar but heat-inactivated serum. The migration inhibitory effect of normal guinea pig serum could be abolished by using complement destroying agents such as: heating at 56°C for only 5 min, absorption on immune complexes, zymosan, and Sephadex G-50, or by adding EDTA as a chelating agent, or heparin, to the culture medium. On the other hand, the migration inhibitory activity of GPS disappeared after *in vitro* absorption on homologous blood cells but the complement haemolytic function remained unaffected.

These preliminary data suggested that the migration inhibitory activity of guinea pig

Abbreviations used in this paper: GPS, Guinea pig serum; HIGPS, Heat-inactivated guinea pig serum; MIF, Migration inhibitory factor; MI, Migration index; SRBC, Sheep red blood cells; PBL, Peripheral blood leucocytes; PBS, Phosphate buffer saline; PEC, Peritoneal exudate cells.

serum is mediated by a factor which requires some of the complement components in order to become fully active.

MATERIAL AND METHODS

Preparation of GPS samples depleted of complement factors

Blood was drawn from outbred guinea pigs by i.c. puncture and incubated for 60 min at 37°C and 90 min at 4°C. Serum was removed and centrifuged at 2000 g for 15 min. Aliquots of GPS were processed as follows:

- a) Heated at 56°C for 5 or 30 min.
- b) GPS and HIGPS were each mixed with 2 mg/ml ovalbumin-rabbit anti-ovalbumin insoluble immune complexes in absence or presence of EDTA 0.01 M final concentration (EDTA, UCB Belgium) and incubated at 37°C for 1 h with occasional shaking. Finally, the suspension was centrifuged at 2000 g for 15 min and the supernatant collected for testing. To the samples absorbed in the presence of EDTA was added 0.01 M calcium chloride final concentration at end of incubation period.
- c) GPS and HIGPS were each mixed with 20 mg/ml zymosan (Serva, Germany) prepared according to Kabat & Mayer's technique (14), incubated for 1 h at 37°C with occasional shaking, and stored overnight at 4°C. After centrifuging at 2000 g for 15 min the supernatant was collected for testing.
- d) GPS and HIGPS were mixed with an equal volume of ether by inverting the tubes for 5 min (14), after which the serum was removed for testing.
- e) Sephadex G-50 (Pharmacia, Sweden) washed with PBS was mixed with GPS and HIGPS at a 20 mg/ml concentration and incubated overnight at 4°C. After centrifuging at 2000 g for 15 min the supernatants were collected for testing.
- f) Pure heparin (Calbiochem) was added to both GPS and HIGPS preparations in a concentration of 10⁻³ g/ml.

- g) GPS and HIGPS were dialysed for 24 h at 4°C on a magnetic stirrer against phosphate buffer ($\mu = 0.02$, pH 5.4) (14). After dialysis the content of the cold and the supernatant fluid was removed. The precipitate was washed twice with cold buffer ($\mu = 0.02$, pH 5.4) and dissolved in PBS. The precipitates and the supernatants prepared from GPS were recombined with their complementary supernatants and precipitates prepared from HIGPS, and each recombinant was tested for migration inhibitory activity. A mixture of the precipitate and the supernatant fractions prepared from HIGPS was used as control.

Absorption of GPS on homologous blood cells

GPS was mixed v/v with homologous pelleted blood cells and incubated overnight at 4°C. The mixture was centrifuged at 2000 g and the supernatant tested for MIF activity. HIGPS was treated similarly and used as control.

Complement titration by haemolysis assay

This was done essentially by using the technique of Mayer (14) and SRBC-anti-SRBC serum system. Titres were expressed as the highest dilution of GPS or its fractions to give complete haemolysis.

Migration inhibition assay

Cell migration from blood clot fragments method. This method was performed as previously described (15). Briefly, from a blood clot, obtained by incubating 2 ml blood at room temperature in a 3.2 cm diam. glass dish, fragments were cut with a 1.2 mm diam. trocar and cultured after several washings in control and assay media.

Cell migration from clotted plasma droplets. In this method a slightly modified technique (16) was used which has been described previously. Initially, the peripheral blood leucocytes were separated by Ficoll-isopaque centrifugation.

Target cells (granulocytes, lymphomonocytes or buffy coat cells) 5×10^5 were suspended in 50 μ l plasma (obtained by centrifuging blood harvested on EDTA 0.01 M final concentration) or in 0.5–1% fibrinogen in saline, and the preparations were cooled on ice. 10 μ l of a 0.1 M CaCl_2 solution and 10 μ l thrombin (0.6–1 U) were added respectively. With a Finnpiptette, droplets of 5 μ l of the cell suspension were placed in the centre of the migration chambers (Sterilin) and the preparations were incubated at 37°C for 20 min in a water-saturated atmosphere. After filling up with control and assay media, the migration chambers were sealed with coverslips and incubated for 16–20 h at 37°C (17).

The capillary tube technique. The capillary tube technique for PEC migration was done as described previously (15).

Quantitative determinations. Quantitative determinations were performed gravimetrically by projecting the migration areas on Whatman paper. Arithmetic mean $\pm$ s.d., Migration Index and *t*-test were determined. MI was calculated as percentage of control. $\text{MI} = (\text{Area X}/\text{Area control}) \times 100$.

RESULTS

In vitro migration of cells from blood clot fragments or from clotted plasma droplets of normal guinea pigs are significantly inhibited by the addition of autologous (homologous) non-inactivated serum to the culture medium, as compared with the same serum, but heat-inactivated. Using varying proportions of GPS mixed with HIGPS, and adding the serum mixture to the culture medium at a final concentration of 15%, the migration inhibitory activity could still be detected even at 5% GPS concentration (Fig. 1).

The migration inhibitory activity is heat labile, being destroyed on heating at 56°C for 5 min, and is lost by absorbing GPS on immune complexes in the presence of calcium, or by absorbing it on zymosan or Sephadex G-50,

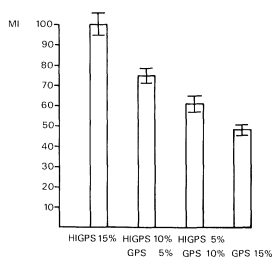


Fig. 1. Influence of various concentrations of GPS upon cell migration from blood clot fragments *in vitro*. HIGPS, heat inactivated guinea pig serum, 30 min at 56°C; GPS, guinea pig serum. MI (Migration Index) was calculated as percentage of control, $(\text{Area X}/\text{Area control}) \times 100$, by using arithmetic mean values of three experiments, each being computed from four to six individual migration area projections on Whatman paper.

concomitantly with complement haemolytic function. When other well-known complement inactivating agents were used, the migration inhibitory effect of both GPS and its complement haemolytic activity disappeared. Pretreatment of GPS with ether, or by adding EDTA or heparin to the culture medium, also abrogated or decreased the migration inhibitory activity of GPS (Fig. 2).

When GPS was separated into an insoluble (euglobulin) and a soluble (pseudoglobulin) fraction by dialysis, both the migration inhibitory effect and the complement haemolytic function of each fraction were lost but restored by recombining the two fractions (Fig. 3).

GPS previously absorbed on homologous blood cells lost its migration inhibitory activity but retained its complement haemolytic function. Recombination of GPS absorbed on homologous blood cells with serum samples inactivated by heating for 5 min at 56°C–

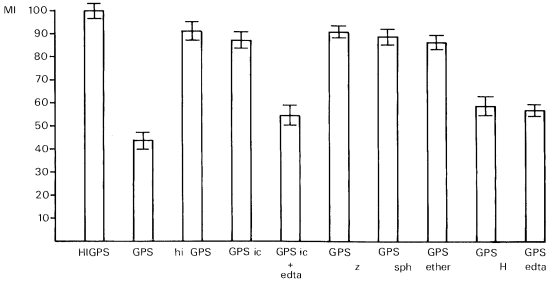


Fig. 2. Action of complement destroying agents upon leucocyte migration inhibitory activity of guinea pig serum. HIGPS, heat-inactivated guinea pig serum, 30 min at 56°C; GPS, non-inactivated guinea pig serum; hiGPS, 5 min heat-inactivated GPS; GPSic, GPS absorbed on ovalbumin-rabbit anti-ovalbumin insoluble immune complexes; GPSic + edta, GPS absorbed on immune complexes as above, but in presence of EDTA 0.01 M; GPSz, GPS absorbed on zymosan; GPSsph, GPS absorbed on Sephadex G-50; GPSether, GPS mixed and shaken with ether, before testing; GPSH, heparin 10⁻² g/ml was added to control and assay media; GPSedta, EDTA 0.01 M (final concentration) added to control and assay media. Migration indices were calculated as percentage of control, using the mean values of three experiments, each being computed from four to six migration area projections.

each of the two fractions initially being inactive upon cell migration—led to complete restitution of the migration inhibitory activity. However, the proportion of the two recombinants is of critical importance. The proportion of the 5-min inactivated serum must be higher than the absorbed one. When the proportion is inverted, no recovery of the inhibitory effect on cell migration could be observed (Fig. 4).

When Ficoll-separated PBL were used as target, by means of the clotted plasma droplet technique, granulocytes appeared to be more sensitive to the migration inhibitory activity of GPS than lymphomonocytes. Also PEC migration from capillary tubes was only moderately inhibited by GPS (Table 1).

DISCUSSION

Autologous or homologous GPS inhibits the *in vitro* migration of cells from blood clot

fragments or from clotted plasma droplets. The inhibitory effect is detectable even at a 5% concentration of GPS in the medium. The magnitude of the inhibitory effect appears to be dependent on the migration system used. Lower values were recorded by the macrophage migration from the capillary tube compared with the blood clot fragment method. By using the clotted plasma droplet technique, granulocytes appear to be more sensitive to the migration inhibitory activity than lymphomonocytes.

A thermolabile MIF, in the lymph of sensitized sheep and pigs, which could be destroyed by heating for 5 min at 56°C, has been previously described (9, 10). Sephadex G-200 or ammonium sulphate fractions of the lymph, which initially lacked MIF activity when tested alone, regained a powerful inhibitory effect upon recombination. This MIF

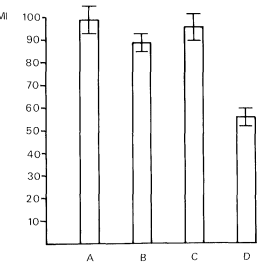


Fig. 3. Reversible loss of migration inhibitory activity of guinea pig serum by dialysation and its recovery by recombination of fractions. Migration indices in presence of: A: Mixture of supernatant and precipitate dialysation fractions of heat-inactivated guinea pig serum (control); B: Precipitate dialysation fraction of GPS + supernatant dialysation fraction of heat-inactivated serum; C: Supernatant dialysation fraction of GPS + precipitate dialysation fraction of heat-inactivated serum; D: Mixture of supernatant and precipitate dialysation fractions of GPS. MI was calculated as percentage of control ($MI = \text{Area X}/\text{Area control} \times 100$), by using the arithmetic mean values of three experiments, each being computed from four to six individual migration area projections on Whatman paper.

activity was related to a preliminary sensitization, and it was claimed that the afferent lymph of the antigenic-stimulated drainage area did not contain demonstrable MIF activity at a time when the efferent lymph activity was strongly inhibitory. Moreover, removal of fibrinogen decreased or abolished the MIF effect. The addition of non-inactivated GPS increased the MIF activity of the efferent lymph obtained after antigenic stimulation (10). Furthermore, Fox et al. (13) found an MIF-like substance in fresh non-inactivated foetal calf serum and they claimed it to be similar to if not identical with MIF secreted by antigenic-stimulated lymphocytes.

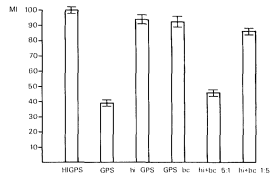


Fig. 4. Loss of migration inhibitory activity of GPS by absorption on homologous blood cells and its recovery by recombination with heat-inactivated serum. HI-GPS (control), heat-inactivated guinea pig serum, 30 min at 56°C; GPS, guinea pig serum; hi-GPS, 5 min heat-inactivated GPS at 56°C; GPS-hi, GPS absorbed *in vacuo* on pelleted homologous blood cells; hi + bc 5:1, recombination of hi-GPS with GPS-hi in proportion of 5v:1v; hi + bc 1:5, the same recombinants as before but with inverted proportions; MI was calculated as percentage of control migration (cell migration in medium enriched with 15% HI-GPS).

In contrast, we find that the inhibitory activity of GPS seems to be related to its complement haemolytic function as it is similarly sensitive to agents known to inactivate specifically some of the complement components. This brought about a loss in the complement haemolytic activity and also of the migration inhibitory effect (Table 1). By heating GPS at 56°C for 5 min, just enough to destroy C_1 and C_2 components (14), the complement haemolytic function as well as the migration inhibitory activity disappeared. The binding of C_3 to immune complexes by absorption experiments, a process requiring calcium ions and which can be preserved by addition of EDTA, leads to the inactivation of both the complement haemolytic and the migration inhibitory activities of GPS. The inactivation of C_3 component by heparin, or by absorbing it with zymosan or dextran, causes a significant decrease of the migration inhibitory effect of GPS concomitantly with the inactivation of its complement haemolytic function. These results indicate that C_3 is also involved in the expression of the migration

Table 1
Migration inhibitory activity of GPS tested on different homologous target cells

Target cell	HIGPS ^d	GPS ^e	MIF
	Migration $\pm$ s.d. ^f	Migration $\pm$ s.d.	
Granulocytes ^a	412.73 $\pm$ 0.8	223.4 $\pm$ 11.29	54.1
Lymphomonocytes ^b	362.13 $\pm$ 6.7	254.1 $\pm$ 5.88	70.1
PEC ^c	188.37 $\pm$ 4.6	135.4 $\pm$ 1.81	71.9

^{a,b} = Granulocytes and lymphomonocytes resp., separated from PBL by Ficoll-isopaque centrifugation were used as target by means of the clotted plasma droplet technique
^c PEC, Peritoneal exudate cells harvested from amidon starch (2.5%), i.p. inoculated guinea pigs were used as target by means of capillary tube technique
^d 15% heat-inactivated guinea pig serum in MEM was used as control
^e 15% fresh guinea pig serum in MEM was the assay medium
^f Migration mean values $\pm$ 1 s.d. of three to four area projections on Whatman paper
^g Migration index was calculated as percentage of control = (Area X/Area control) $\times$ 100.

inhibitory action of GPS. The inactivation of C₁ component by ether treatment of GPS (14) causes the disappearance of both complement haemolytic and migration inhibitory activities. Moreover, on GPS dialysation, leading to the separation of the complement components, C₁ and C₂ remaining in the insoluble fraction and C₃ and C₅ in the supernatant fluid (14), the migration inhibitory activity of each fraction disappeared. The recombination experiments with the euglobulin and the pseudoglobulin fractions of GPS, suggest that all four first components of complement are necessary for both the complement haemolytic and the migration inhibitory functions.

On the other hand, by absorbing GPS on homologous blood cells only the migration inhibitory capacity disappeared leaving the complement haemolytic function unimpaired. This demonstrates that migration inhibitory activity could be removed from GPS without affecting the complement haemolytic function.

In contrast, inactivation of complement leads concomitantly to the disappearance of the migration inhibitory activity of GPS suggesting that some of the complement factors are necessary for the expression of this activity. GPS treated at 56°C for 5 min has no

complement activity because of C₁ and C₂ destruction and no migration inhibitory activity, although the inhibitory factor could be preserved in a non-active state. GPS absorbed on homologous blood cells exhibits only the complement haemolytic function. When five parts of GPS treated at 56°C for 5 min are mixed with one part of GPS absorbed on homologous blood cells, the migration inhibitory activity is regained. This suggests that this inhibitory factor is present in GPS samples treated at 56°C for 5 min (but not at 30 min) and can be activated by complement components present in serum samples absorbed on homologous blood cells. The failure to inhibit cell migration when the proportion of the recombinants was inverted, confirmed this possibility. Similar findings have been obtained with recombinants of GPS samples absorbed on immune complexes and those absorbed on homologous blood cells (data not presented). Our data are more similar to recent findings demonstrating both the role of complement factors in macrophage spreading (18) and the production of an MIF-like activity in GPS by incubation with plasminogen activator produced by Simian virus 40 transformed mouse 3T3 cells (19).

In conclusion, the migration inhibitory activity of GPS evidenced by our experiments appears to be a multifactorial function which is at least partially absorbable on homologous blood cells and requires some complement factors for its activation. New investigations are necessary to clarify its nature.

ACKNOWLEDGEMENTS

We thank Dr. V. Gheie at the Victor Babes Institute, Bucharest, Rumania, for his help in carrying out the serum dialyses and absorptions, during Dr. G. Sandru's stay at this Institute. We acknowledge the excellent technical assistance of Alexandru Sergiu.

REFERENCES

1. Bloom, B. R. & Bennet, B.: Mechanism of a reaction in vitro associated with delayed type hypersensitivity. *Science* **153**, 80-82, 1966.
2. Amos, H. E. & Lachmann, P.: The immunological specificity of a macrophage inhibition factor. *Immunology* **16**, 269-278, 1970.
3. Remold, H. G., Haber, E. & David, J. R.: Cellular hypersensitivity studies on the purification of migration inhibitory factor (MIF). In Sierl, J. & Riha, J. (eds.): Developmental aspects of antibody formation and structure, Vol. 1, p. 179. Academia Prague and Academic Press, New York and London, 1970.
4. Yoshida, T. & Reisfeld, R. A.: Two fractions with macrophage migration inhibitory activity from sensitized lymphocyte cultures. *Nature (Lond.)* **226**, 856-857, 1970.
5. Spitzer, L., Haber, H. & Fudenberg, H. H.: Induction of capillary migration by antigen-antibody complexes. *J. Immunol.* **102**, 404-411, 1969.
6. Amos, H. E., Gurner, B. W., Olds, R. J. & Coombs, R. R. A.: Passive sensitization of tissue cells. II. Ability of cytophilic antibody to render the migration of guinea pig peritoneal exudate cells inhibitable by antigen. *Int. Arch. Allergy* **32**, 496-504, 1967.
7. Hebe, E. R.: In Pearsall, N. N. & Weiser, R. S. (eds.): *The Macrophage*, p. 109. Lea and Febiger, Philadelphia, 1970.
8. Stasny, P. & Ziff, M.: Inhibitor of macrophage migration produced by polymorpho-nuclear leucocytes. *J. Reticuloendothel. Soc.* **7**, 140-145, 1970.
9. Hay, J. B., Lachmann, P. J. & Trnka, Z.: The appearance of migration inhibition factor and a mitogen in lymph draining tuberculin reactions. *Eur. J. Immunol.* **3**, 127, 1973.
10. Meade, C. J., Lachmann, P. J. & Binns, R. M.: A mechanism of migration inhibition involving components of the coagulation system. *Scand. J. Immunol.* **5**, 529-539, 1976.
11. Granger, G. A., Moore, G. E., White, J. C., Matzinger, P., Sundsmo, J. S., Shupe, S., Kolb, W. P., Kramer, J. & Glade, P. R.: Production of lymphotoxin and migration inhibitory factors by established human lymphocytic cell lines. *J. Immunol.* **104**, 1476-1485, 1970.
12. Papageorgiou, P. S., Henley, W. L. & Glade, P. R.: Production and characterization of migration inhibitory factors (MIF) of established lymphoid and non-lymphoid cell lines. *J. Immunol.* **107**, 494-504, 1972.
13. Fox, R. A., Gregory, D. S. & Feldman, J. D.: Migration inhibition factor (MIF) and migration stimulation factor (MSF) in foetal calf serum. *J. Immunol.* **112**, 1861-1866, 1974.
14. Mayer, M. M.: In Kabat, E. & Mayer, M. M. (eds.): *Experimental Immunochimistry*, 2nd edn., pp. 133, 159, 160, 163. Charles C Thomas, Springfield, 1961.
15. Sandru, G.: A leukocyte migration inhibition assay technique using blood clot fragments in vitro. *Eur. J. Immunol.* **5**, 729-731, 1975.
16. Sandru, G. & Sandru, L.: Cell migration from clotted plasma droplets in vitro: Development of a new method for MIF assay. *J. Immunol. Methods* **19**, 293-298, 1978.
17. Sandru, G. & Verguth, P.: Technical improvements in the clotted plasma droplets MIF assay in vitro. *J. Immunol. Methods* 1979 (in press).
18. Goetze, O., Bianco, C. & Cohn, Z. A.: The induction of macrophage spreading by a factor B of the properdin system. *J. Exp. Med.* **149**, 372-386, 1979.
19. Roblin, R. O., Hammond, M. E., Bensky, N. D., Dvorak, A. M., Dvorak, H. F. & Black, P. H.: Generation of macrophage migration inhibitory activity by plasminogen activators. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1570-1574, 1977.

Address:

Dr. G. Sandru
Laboratory of Radiobiology
Dept. of Radiotherapy
University of Berne
Inselspital, 3010 Berne
Switzerland

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.