Clin. exp. Immunol. (1977) 29, 181–186.

METHODS

Migration inhibition factor and the blood clotting system: effects of defibrination, heparin and thrombin

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(*Received* 31 *January* 1977)

SUMMARY

Heparin is not suitable as an anticoagulant in the leucocyte migration test used to demonstrate the presence of migration inhibition factor (MIF) due to a rapid disappearance of the response after even a short storage of the blood.

The use of defibrinated blood is highly recommended and defibrinated blood can be stored for at least 90 min without any diminution of the response. The change in response when using heparinized blood is not due to any direct effect of heparin, because heparin has no effect when added to defibrinated blood. However, heparin, added together with thrombin, is capable of abolishing the MIF effect completely. The basis for this phenomenon is most probably the binding of the heparin–antithrombin cofactor (AT III) to a complex with heparin and thrombin. The activity of MIF requires the presence of AT III, its esterase-inhibiting activity probably being crucial, in order to express MIF activity on macrophages. This mechanism forms a link between certain cellular immune reactions and the blood-clotting system.

INTRODUCTION

Sensitized thymus-derived lymphocytes (T lymphocytes), exposed to corresponding antigens, produce a number of mediators called lymphokines, one of which is the macrophage MIF. The leucocyte migration test (LMT), as first described by Søborg & Bendixen (1967), has been widely used for the demonstration of the presence of MIF. This method is probably the most sensitive *in vitro* technique for the detection of cell-mediated immunity (CMI). The method has been employed, with various modifications, in the following conditions: microbial hypersensitivity, autoimmune diseases, drug hypersensitivity, tumour immunity and transplantation reactions (Søborg, 1971). Although most investigators have obtained satisfactory results with the LMT, certain technical problems exist and have been discussed (Bendixen & Søborg, 1970).

During our studies on CMI with the LMT in different autoimmune thyroid diseases (Tötterman, Mäkinen & Gordin, 1977) it was observed that the effect of MIF markedly decreases upon storage of heparinized blood. This effect was attributed to the presence of heparin and consequently defibrinated blood was tested. The present investigation was undertaken in order to elucidate factors affecting the outcome of the LMT. In addition to studying the effect of thyroid antigen in thyroid patients, we also studied purified-protein derivative (PPD) in BCG-vaccinated subjects, as a general test for CMI *in vitro*. The effect of thrombin was also studied in the light of a recent report stressing the importance of various esterase inhibitors as enhancing factors for MIF in the guinea-pig (Remold & Rosenberg, 1975).

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MATERIALS AND METHODS

The leucocyte migration test. The LMT according to Søborg & Bendixen (1967) was performed with slight modifications. Blood samples (20 ml) were either defibrinated with glass beads or anticoagulated with 20 iu preservative-free heparin (Medica, Finland) per ml of blood. The blood samples were then mixed with 4 ml of a 6% dextran (Pharmacia, Sweden, mol. wt. 250,000) solution and allowed to sediment for 1 hr at 37°C. The leucocyte-rich plasma was collected and the remaining erythrocytes removed by treatment with 0·84% NH_4Cl solution. The leucocytes were washed three times in 5 ml of Hank's balanced salt solution and finally suspended in tissue culture medium TC-199 (Difco, U.S.A.), pH 7·3, supplemented with 10% (v/v) horse serum. The final cell concentration was $2{\cdot}5 \times 10^8$ leucocytes per ml. 20 1 μl capillary glass tubes (Drummond, U.S.A.) were filled with the leucocyte suspension, sealed at one end with wax (Clay Adams, England) and centrifuged at 900 *g* for 10 min.

The tubes were then cut about 1 mm below the cell-medium interface and immediately placed into plastic chambers (Sterilin, England), containing 0·5 ml of culture medium with, or without, added antigens. The chambers were sealed with coverslips (Chance Propper, England) and incubated for 20 hr at 37°C. Each test was run in triplicate. The migration areas were magnified, measured by planimetry and the results calculated using the formula:

$$\text{migration index (MI)}\ (\%) = \frac{\text{mean area of migration with antigen}}{\text{mean area of migration without antigen}} \times 100.$$

Thyroid antigen. Antigen was prepared from a surgically removed gland from a patient with toxic diffuse goitre. The gland was homogenized in cold phosphate-buffered saline (pH 7·2) with a Potter–Elvehjem apparatus. The homogenate was filtered through a nylon cloth and subsequently lyophilized. The protein concentration was determined by the method of Lowry *et al.* (1951) using human serum albumin as standard. Antigen was added in a final concentration of 360 μg protein/ml of medium.

Purified-protein derivative (preservative-free PPD). This antigen was obtained from the State Serum Institute, Copenhagen, Denmark. PPD was added to the medium in a final concentration of 5000 iu/ml.

Thrombin. Bovine thrombin (Topostasin®) was obtained from Hoffmann–La Roche & Co., Switzerland, and added to the blood samples in a final concentration of 4 NIH or 0·08 mg per ml of blood.

The controls. Twelve apparently healthy subjects were tested with thyroid antigen. None of them had a present or past history of thyroid disorders, and they did not have goitre. All the controls were tested using both heparin and defibrination in the assay.

The thyroid patients. The patients tested with thyroid antigen consisted of seven cases with Graves disease, two with toxic nodular goitre and six with primary spontaneous hypothyroidism. These patients were part of a larger study from our laboratory. Ten of these patients were tested using both heparin and defibrination, in the experiment when the leucocytes were separated immediately after anticoagulation. The effect of storage of the blood was tested in five patients using heparin and in four patients using defibrination. Since quite substantial amounts of blood had to be drawn, the two tests were not done in parallel in the same patient.

The tuberculin-positive test subjects. Eleven healthy subjects were examined with PPD. All had been vaccinated with BCG and found positive in skin tests. Seven of these were tested using both heparin and defibrination when the assay was started immediately after separation of the leucocytes. The effect of storage of the blood was tested in seven subjects with heparin and seven with defibrination. Furthermore, the effect of heparin and thrombin added to defibrinated blood was tested in three subjects.

Statistical methods. The paired *t*-test was used for testing the significance of differences in pairs of migration indices.

RESULTS

Characteristics of the LMT

The mean MI for the twelve healthy controls tested with thyroid antigen was $93 \pm 3\%$ (mean $\pm$ s.d.) when defibrinated samples were assayed immediately after separation of leucocytes. The normal range of migration using defibrination was taken as 87–99%, i.e. ± 2 s.d. from the mean. When heparin was used as anticoagulant the mean was $95 \pm 4\%$ and the normal range of migration 87–103%. The means did not differ significantly ($P > 0{\cdot}05$). The intra-assay coefficient of variation between triplicates was 4·2%. The inter-assay coefficient of variation was 3·4%, when the same subject was tested for PPD sensitivity nine times during a period of 8 months.

Effect of anticoagulation with heparin vs defibrination

No storage of the blood. When ten thyroid patients were tested with thyroid antigen (the leucocytes

were separated immediately after anticoagulation), the mean MI in the defibrinated samples was 77±9% and in the heparin-treated samples 80±8%. This difference was almost significant using the paired *t*-test ($P < 0.05$) (Table 1). When seven BCG-vaccinated subjects were tested with PPD, the mean MI with defibrination was 67±12% and the mean with heparin 71±11%. Again, the difference was almost significant ($P < 0.05$) (Table 1).

TABLE 1. Comparison of the LMT with heparinized or defibrinated blood processed immediately after collection

	MI (%)	
(a) Thyroid patient no.	Heparin	Defibrination
1	71	74
2	72	66
3	72	71
4	72	62
5	79	77
6	80	75
7	80	81
8	87	86
9	88	86
10	95	90
(b) BCG-vaccinated subject no.		
1	53	48
2	64	56
3	64	64
4	75	70
5	77	75
6	80	75
7	82	82

(a) = Thyroid antigen, (b) = PPD.

Thus the response was enhanced in defibrinated blood, in some cases, with both antigens.

Effect of storage of the blood. The effect of storage of heparinized or defibrinated blood is shown in Fig. 1. As can be seen, 90 min storage of the heparinized blood at 20°C before separation of the leucocytes had a marked inhibiting effect on the activity or production of MIF. This holds true both for patients reacting to thyroid antigen (Fig. 1(a)) and to persons reacting to PPD (Fig. 1 (b)), the reduction of the response being about 10–15%. On the other hand, defibrinated blood could be stored for at least 90 min without any detectable effects at all on the MIF effect using both antigens (Fig. 1).

Effects of heparin and thrombin on MIF

In order to study the effect of heparin more in detail, three subjects reacting to PPD were chosen for further tests. These results are summarized in Fig. 2. Heparin was added to defibrinated blood (20 iu/ml), which was subsequently stored for 90 min. However, the addition of heparin to defibrinated blood had no effect, or a very slight one, on the MIF compared to the corresponding sample without heparin. It was concluded that heparin, together with some cofactor lacking from defibrinated blood, was able to inhibit the LMT response. Therefore the effect of heparin and thrombin added together to defibrinated blood was examined. When thrombin in physiological amounts (4 NIH/ml) was added

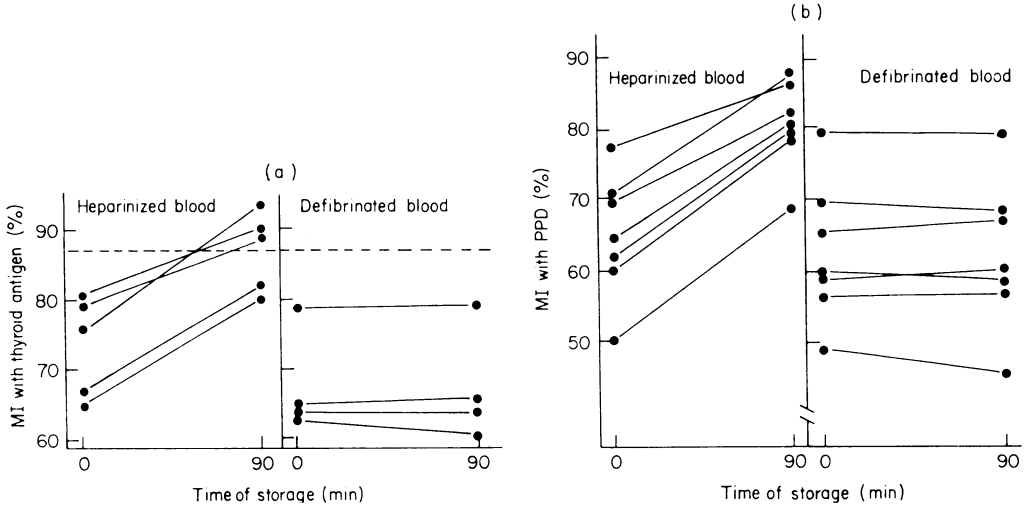

FIG. 1. Effect of storage of heparinized or defibrinized blood samples on the leucocyte migration test performed with thyroid antigen in thyroid patients (a) or PPD in BCG-vaccinated subjects (b). The broken line marks the lower normal limit for the test.

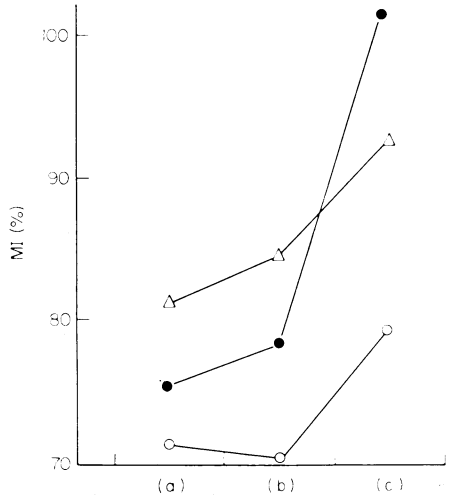

FIG. 2. Effect of addition of heparin alone, or heparin and thrombin combined, to defibrinated blood used in the leucocyte migration test: (a) defibrinated blood without additions; (b) defibrinated blood with 20 iu heparin per ml added; and (c) defibrinated blood with 20 iu heparin and 4 NIH thrombin per ml added. The different symbols denote three subjects examined using PPD as antigen. The blood was stored for 90 min before the leucocytes were separated.

together with heparin, the effect of MIF was almost completely abolished. Thrombin added alone without heparin to defibrinated blood had no significant effect on the LMT.

DISCUSSION

The present investigation clearly points out the need for strict standardization of the experimental procedure, beginning with the taking of the blood samples, when the LMT is performed. Heparinized blood is very sensitive to even a short storage prior to isolation of leucocytes, since the specific inhibition disappeared even after a 90 min storage of the samples before processing of the cells. On the other hand,

defibrinated blood could be stored for at least this time without any detectable effect on the LMT. This is true both for thyroid antigen in patients with thyroid autoimmune disease and PPD in BCG-vaccinated persons. In our opinion heparin is not suitable as an anticoagulant in tests of this type and should be avoided. Defibrination seems to be the method of choice, since no external factors of unknown biological effect are added.

The deleterious effect of heparin on the LMT is not a direct one, as heparin added to defibrinated blood was without any effect at all. Thus it seems likely that heparin needs one or several factors lacking from defibrinated blood, but existing in native blood. During blood clotting thrombin is absorbed by the newly formed fibrin and thereby removed from the blood (Seegers, 1973). It is known that the action of heparin in preventing blood clotting is complex, but it acts as an antithrombin and also inhibits formation of thromboplastin. The antithrombin action of heparin needs the presence of the antithrombin–heparin cofactor, i.e. the antithrombin-III factor (AT III) (Seegers, 1973; Abildgaard, 1969). AT III is known to be an esterase inhibitor and probably has a significant physiological role in preventing intravascular activity of thrombin. It has been reported that the MIF activity is greatly enhanced by such esterase inhibitors (Remold & Rosenberg, 1975), and it could well be assumed that the presence of certain plasma-esterase inhibitors is a prerequisite for the action of MIF on macrophages.

In our investigation it is demonstrated that heparin together with added thrombin is capable of completely abolishing the effect of MIF on macrophages, while neither substance alone has any effect. One hypothetical reason for these effects of thrombin and heparin combined could be that when heparin is added to normal plasma it prevents clotting by acting as an antithrombin. For this effect the presence of AT III is needed and the AT III–heparin–thrombin probably will form a complex. When heparin is added to defibrinated blood this complex formation does not occur, since thrombin is lacking and AT III remains in an active state, which is also the case when thrombin without heparin is added to the defibrinated blood. The slight and mostly non-significant effect of thrombin or heparin alone is probably due to a weak or different complex formation with a part of the AT III, leaving most of its esterase-inhibiting activity unaltered. We therefore postulate that the action of MIF needs the presence of AT III. The evidence is indirect, however, because purified AT III was not available for use in our study, neither were measurements of esterase-inhibitor concentrations made. A study on these topics would be highly rewarding.

In the LMT the white cells are washed three times before placing them in the horse-serum-containing culture medium. It is likely that most of the soluble plasma factors are removed, and that only cell-associated esterase inhibitors are present during the overnight incubation. In heparin-treated whole blood presumably a macrophage-associated heparin–thrombin–AT III complex is formed, whereby the action of MIF on the macrophage is abolished. The horse serum probably contains esterase inhibitors in varying amounts, but their role in the LMT is unclear. In any case, some sera constantly give negative LMTs, and sera have to be selected for the test. In our study several batches from different horses gave remarkably similar MIs for the same person, repeatedly tested, for PPD sensitivity over a period of 8 months.

It has been shown with guinea-pig macrophages that AT III of human origin greatly enhances the effect of MIF (Remold & Rosenberg, 1975). In the system using guinea-pig macrophages this effect was blocked by heparin, which is not the case in our system using human cells. However, this difference might well be due to some technical details and does not speak against the assumption that AT III plays an important role in certain cellular immune reactions, as exemplified by the LMT. It seems quite reasonable to assume that AT III also has a regulatory function on macrophage activity *in vivo*. Macrophages are rich in esterases, but their biological significance is not known. AT III is likely to have an inhibitory effect on macrophage esterases. Whether this effect protects the MIF from destruction by esterases or directly affects the macrophages themselves is not clear. However, one physiological function of AT III in the inflammatory response could be that it enhances the macrophage response to MIF at places where leakage of proteins from the blood into tissue occurs as a consequence of an inflammatory reaction.

It is known that MIF not only inhibits macrophage mobility, thereby preventing them from leaving

the site of inflammation, but also activates them, as reflected by enhanced phagocytosis, glucose oxidation and bacteriostasis (Nathan, Karnovsky & David, 1971).

In conclusion, we have shown that some plasma factors involved in the blood coagulation system are of basic importance for certain cell-mediated immune systems. The antithrombin–heparin cofactor (AT III) is probably one of these plasma factors and appears to be required for the expression of MIF activity *in vitro*. We therefore recommend the use of defibrination for anticoagulation as a first step in the LMT.

This work was supported by grants from the Sigrid Jusélius Foundation and the Finska Läkaresällskapet.

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