J. Biosci., Vol. 22, Number 1, January 1997, pp 59-68. © Printed in India.

Generation of human class I major histocompatibility complex activating factor in serum free medium and its partial characterization

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MS received 24 July 1996; revised 1 October 1996

Abstract. Human peripheral blood mononuclear cells (PBMCs) activated with Con-A release a soluble factor which augments the expression of class I major histocompatibility complex (MHC) antigens by a variety of tumour cells. Previous attempts to purify this factor called MHC-activating factor (AF) (MHC-AF) made us realize that the presence of large numbers and quantities of irrelevant fetal calf serum proteins in the culture supernatants of the activated human PBMCs, interfered with the purification procedure. It was therefore necessary to standardize the use of a serum free culture medium to generate human MHC-AF. In the present communication we have tried several types of culture media and have identified DCCM-2 as the most suitable culture medium to generate human MHC-AF. MHC-AF generated in DCCM-2 medium appears to be a protein molecule resistant to pH 2 treatment but sensitive to heat treatment (56°C × 45 min) and treatment with proteolytic enzymes trypsin and chymotrypsin.

Keywords. MHC antigens; cytokine; serum free medium; MHC-AF

1. Introduction

We have previously shown that mitogen activated human peripheral blood lymphocytes (HPBLs) secrete a factor which augments the expression of class I MHC antigens on tumour cells (Saxena *et al* 1992,1996). Our attempts to purify this factor were hampered by the presence of large quantities of fetal calf serum derived irrelevant proteins present in the culture medium used to activate the PBLs (Saxena *et al* 1996). In order to devise a suitable purification strategy, it was necessary to activate HPBLs in serum free culture medium. In the present study, we have attempted to define culture conditions for generating human MHC-AF in culture media containing low levels of protein. Culture media examined for this purpose included RPMI-1640 medium alone or supplemented with 5% fetal calf serum (FCS) (control), 2% FCS or 1 mg/ml BS A. Two commercially available serum free media DCCM-1 and DCCM-2 were also examined. Our result indicate that DCCM-2 medium which had the minimum amount of proteins (126 μg/ml of defined proteins) supported significant levels of mitogen induced proliferation of HPBLs. MHC-AF was found to be released by activated HPBLs in DCCM-2 medium, as assessed by augmentation of MHC I antigens on HR tumour cells. Unlike IFN-*y*, MHC-AF produced in DCCM-2 medium, was resistant to pH 2·0 treatment. Upon concentration on Amicon 30K membrane, most MHC-AF

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activity was retained, indicating that the molecular weight of MHC-AF could be above 30K. Human MHC-AF was also found to be sensitive to heat treatment and is deactivated by treatment with proteolytic enzymes.

2. Materials and methods

2.1 *Culture medium*

Several types of culture media were used in this study. Tumour cell lines were maintained in RPMM64O from Sigma, supplemented with 5% FCS, 2×10^{-5} M 2-mercaptaethanol (ME), 300µg/ml glutamine, and 6Oµg/ml gentamicin (complete medium). For proliferation assay, RPMI-1640 was supplemented with either 5% FCS, 2% FCS or lmg/ml BS A. Two commercially available serum free media, DCCM-1 and DCCM-2 from Biological Industries, Israel, were also used for proliferation assay and for generating human MHC-AF.

2. 2 *Preparation of crude human MHC-AF*

Human peripheral blood lymphocytes (HPBLs) were derived from blood from normal donors (Red Cross Society, New Delhi, Courtsey Dr M L Gupta), by Ficoll Hypaque density gradient centrifugation at 200 g for 30 min. Cells were washed three times with ice cold PBS, suspended in culture media at 5×10^6/ml and cultured for 3 days with Con-A (5 mg/ml). After 3 days the supernatant was collected by centrifugation at 400 g for 20 min. The proteins in the supernatant were precipitated by adding 599 g/liter of ammonium sulphate (4°C overnight). The precipitated proteins were pelleted by centrifugation at 10,000 g for 20 min., resuspended in deionized water, dialyzed against 0·1 M glycine-HCl buffer, pH 2·0 for two days with three changes and then neutralized to pH 7·0 by using 1O N NaOH. Any insoluble material at this stage was removed by centrifugation. If required the solution was concentrated by using a 30 K Amicon membrane filter.

2.3 *Estimation of cell surface antigen on tumour cell lines*

The tumour cells were cultured at 3×10^5 cells/ml in RPMI-1640 culture medium supplemented with 10% FCS, in the presence ofdesired concentration of the test agents for 2 to 3 days, in 96 Dell flat bottomed culture plates. At the end of the incubation, the cells were harvested and stained for class IMHC antigens (Saxena *et al* 1996). Culture supernatants of HB95 hybridoma were used as a source of a mouse monoclonal antibody reactive to a non-polymorphic region of class I MHC antigens in case of human cell lines. Control or treated cells were suspended in 50 µl of respective hybridoma supernatants and incubated for 20 min at 4°C. Cells were washed once with cold PBS and suspended in 50 µl of rabbit anti mouse Ig FITC at 1:400 dilution. After 20 min incubation at 4°C, cells were washed once with cold PBS and fixed in 0.5 ml of 1% (w/v) paraformaldehyde in PBS. Stained and fixed cells were analysed on a flow-cytometer (Coulter Epics XL model).

2.4 *Proliferation assay*

HPBLs were cultured in 5 different media, RPMI-1640 + 2% FCS, RPMI-1640+ lmg/ml BSA, RPMI-1640 + 5% FCS, DCCM-1 and DCCM-2, at 2×10^5 cells/ml, alone or with Con-A (2 μg/ml) or PHA (2 μg/m1) in 96 well flat bottomed microtiter plates. After 3 days, the cells were pulsed with O·5 μCi [^{3}H] thymidine (methyl-T thymidine, from BRIT, Bombay) per well, for 18 h. Cells were harvested by an automated cell harvester (PHD cell harvester, Cambridge Technology) onto glass fibre discs and deposited in scintillation vials. After drying of discs, 1 ml scintillation cocktail was added per vial and these were kept overnight in dark. The incorporated [^{3}H] thymidine was determined as counts per million by using a Beckmann or LKB Beta liquid scintillation counter.

2.5 *Treatment with proteolytic enzymes coupled to Sepharose beads*

Sepharose CL-48 beads were activated by CNBr activation (Puri 1996). Activated beads were transferred to the solutions of trypsin or chymotrypsin (2·5 mg/ml) and suspension incubated overnight at 4°C. Amount of protein attached to the beads was estimated from the protein concentration before and after the coupling reaction. Enzyme coupled to beads was estimated to be 1·5 mg (trypsin) and 1·4 mg (chymotryp sin) per ml of beads. MHC-AF preparations were incubated with or without enzyme coupled beads (80 mg/ml MHC-AF) for 2h at 37°C. After the enzyme reaction, beads were removed by centrifugation and MHC-AF preparations assessed for biological activity.

3. Results

3.1 *Mitogenesis of HPBLs in different media*

Activation of HPBLs in presence of Con-A or PHA was carried out in culture medium supplemented with FCS as well as serum free medium. Figure 1 shows the rate of proliferation of mitogen activated HPBLs in different culture media. DCCM-1 and DCCM-2 are two commercially available serum free media. DCCM-1 and RPMI-164O supplemented with FCS or BSA have protein in the range of 1–4 mg per ml while in DCCM-2 there is only 126 mg of well defined protein. But the rate of proliferation of HPBLs in all the media is comparable. Since DCCM-2 medium was supportive of the proliferative activity in response to mitogens, we further investigated whether this serum free medium also supported the generation of MHC-AF by activated HPBLs.

3.2 *Generation of MHC-AF activity by HPBLs cultured in DCCM-2*

Culture supernatants of HPBLs activated in DCCM-2 medium, were processed as described in §2, and their effect on the expression of class I MHC antigens on human HR cell line, was examined. Control and supernatant treated tumour cells were stained for class I MHC antigens and examined on a flowcytometer. Results

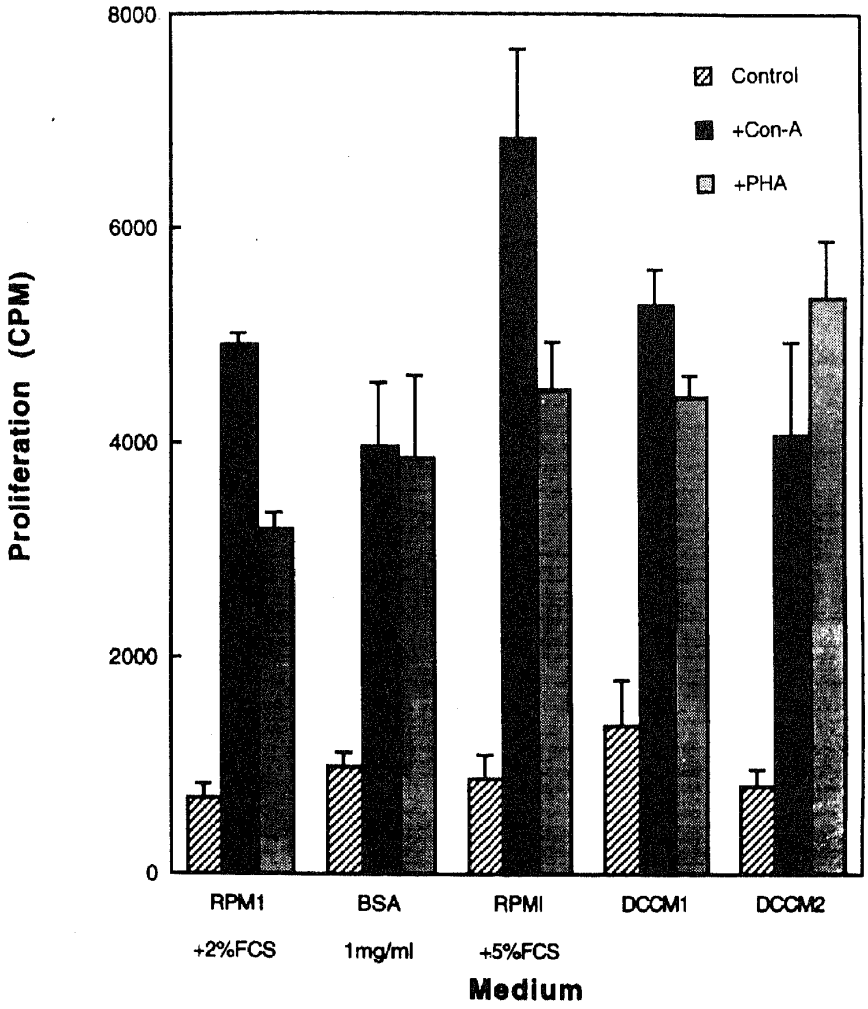


Figure 1. Human peripheral blood lymphocyte (Hu-PBL) mitogenesis in different culture media. Hu-PBLs were cultured in different media, RPMI-1640 + 2% FCS, RPMI-1640+1 mg/ml BSA, RPMI-1640 + 5% FCS, DCCM-1, and DCCM-2, at 5 x 10^6 cells per ml, alone, or with Con-A (2 µg/ml) or PHA (2 µg/ml). [^{3}H] thymidine pulse was given on day 3 and incorporated CPM determined. Mean CPM ± SD of three assay wells, are shown.

in figure 2 show that concentrated culture supernatants generated by using DCCM-2 medium, brought about a marked dose dependent increase in the expression of class IMHC antigens on HR cells. MHC-AF activity is thus secreted by HPBLs activated in DCCM-2 serum free culture medium.

3.3 *Molecular weight of MHC-AF*

To get an idea about molecular weight of MHC-AF, the Con-A supernatant was concentrated on 30 K Amicon membrane. HR cells were treated with Amicon concentrated MHC-AF preparation, fall through fraction of MHC-AF through 30 K

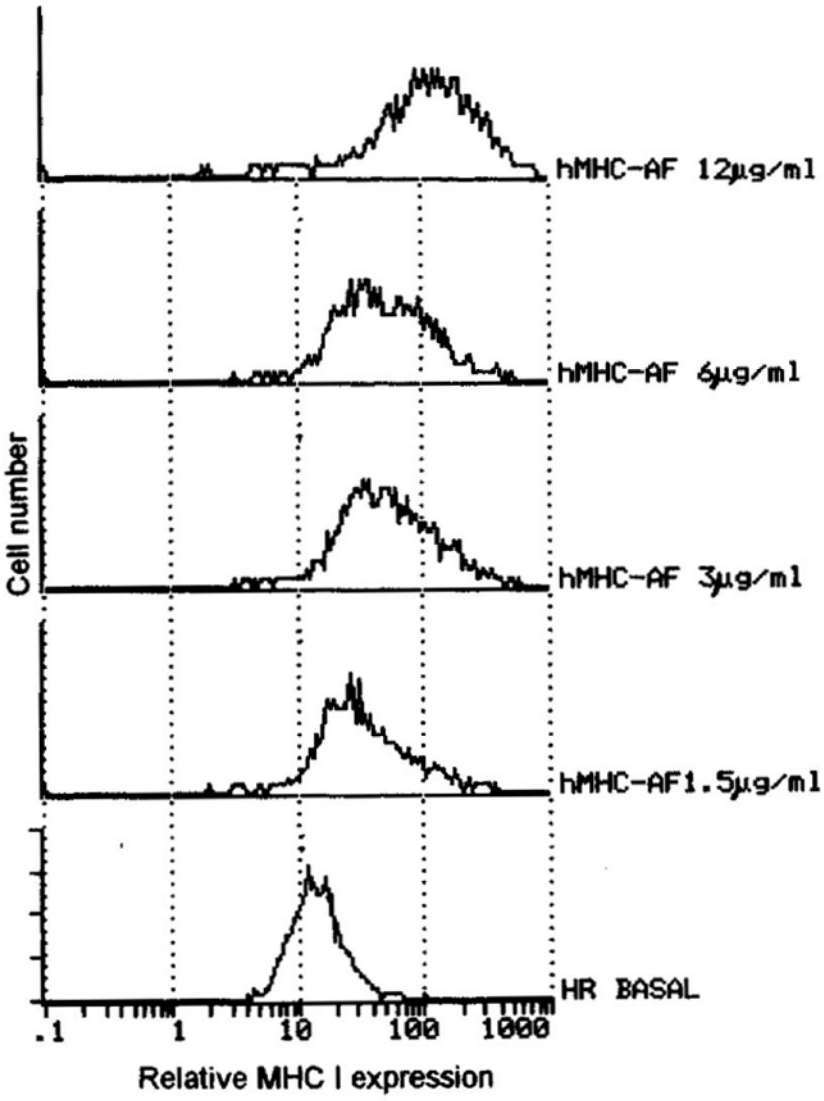


Figure 2. Dose dependent increase in class I MHC expression in HR cell line by human MHC-AF. Culture supernatants ofCon-A activated HPBLs was processed as described in §2. HR cells were cultured at 2×10^5 cells/ml for 72 h at 37°C in 5% CO2 incubator, with different doses of a post dialysis (0·1 M glycine-HCl buffer pH 2·0) MHC-AF preparation, stained for class I MHC expression, and analysed on flowcytometer.

membrane, as well as unfractionated MHC-AF, and efect on MHC I expression was studied. As seen in figure 3, most MHC-AF activity was retained on Amicon 30 K filter,indicating that the molecular weight of MHC-AF could be above 30 kDa.

3.4 *Heat susceptibility of human MHC-AF*

MHC-AF preparations were incubated at different temperatures for 45 min, and then tested for biological activity. Results in figure 4 indicate that treatment at 56°C or

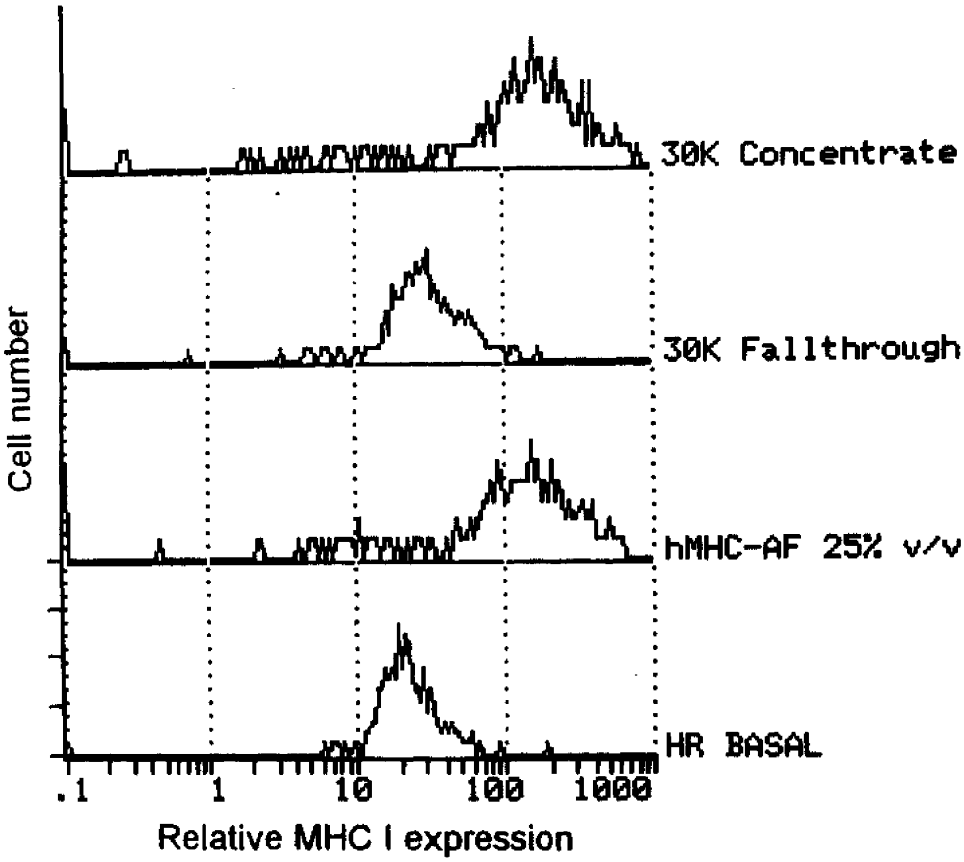


Figure 3. Concentration of human MHC-AF on 30 K Amicon membrane. Crude human MHC-AF was concentrated on 30 K Amicon membrane. HR cells were treated with unfractionated, 30 K concentrate and the 30 K fall through preparations of MHC-AF, all at 25 % v/v. After 3 days incubation at 37°C in 5% CO_2 incubator, HR cells were stained for class I MHC antigens and analysed on flowcytometer.

above for 45 min abrogated the biological activity of MHC-AF. MHC-AF activity is therefore heat labile.

3.5 *Sensitivity of MHC-AF to proteolytic enzymes*

In order to examine the susceptibility of MHC-AF activity to proteolytic enzymes, MHC-AF preparations treated with trypsin and chymotrypsin were tested for biologi cal activity. Presence of proteolytic enzymes in the test preparations was however found to interfere with MHC-AF bioassay. In order to overcome this problem, we treated the MHC-AF preparations with proteolytic enzymes trypsin and chymotrypsin coupled to Sepharose beads (Puri 1996), and the beads were removed by centrifugation prior to bioassay. Treatment of MHC-AF with proteolytic enzymes resulted in a loss of

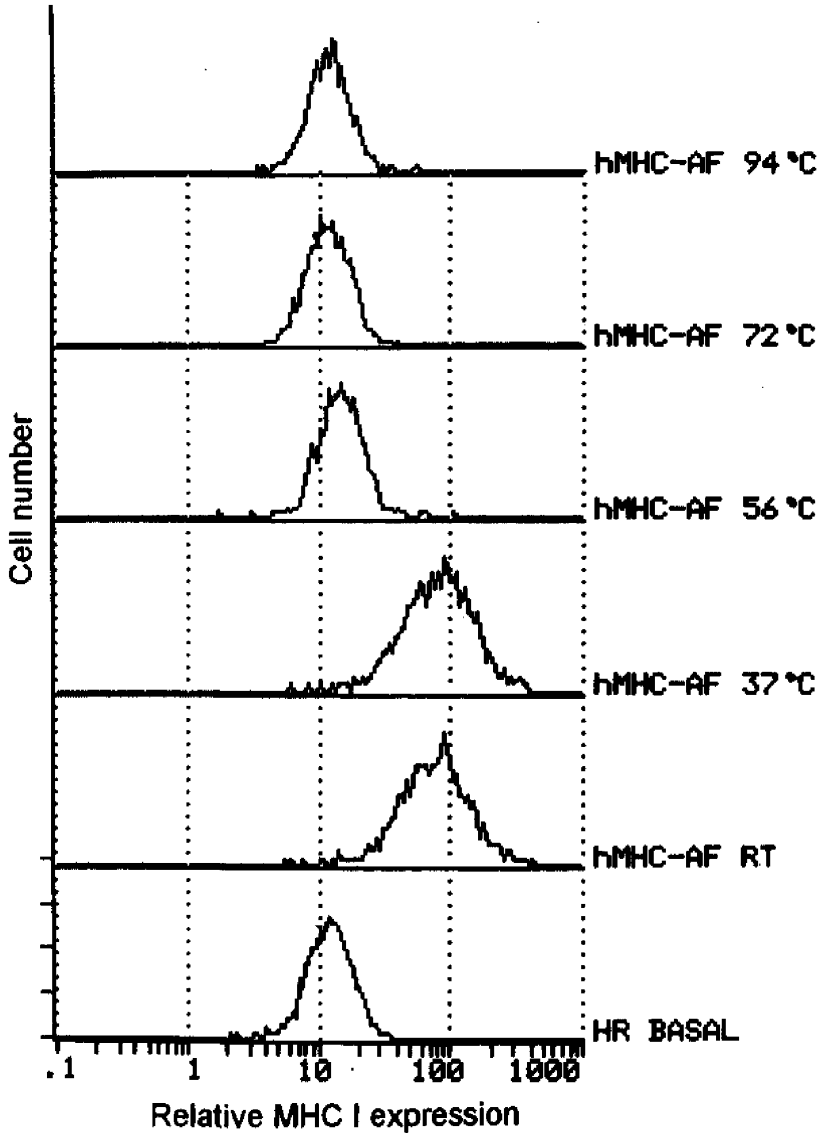
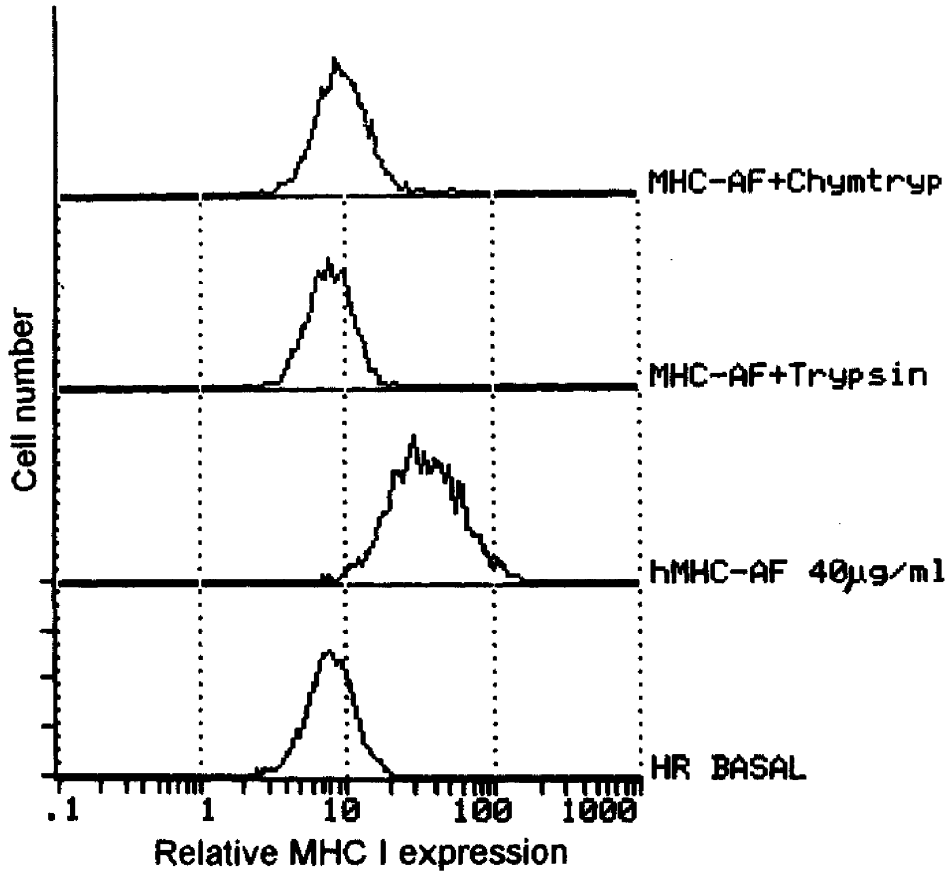


Figure 4. Heat susceptibility of MHC-AF. Aliquotes of MHC-AF preparation were incubated at various given temperatures for 45 min, and examined for biological activity as described in legend to figure 2.

biological activity (figure 5). These results indicate that the MHC-AF molecule is proteinic in nature.

4. Discussion

A soluble factor which induces an augmented expression of class I MHC antigens on a variety of tumour cell lines, is known to be released by mitogen activated lymphocytes in the rat (Saxena 1987; Saxena *et al* 1988), mouse (Saxena *et al* 1992) and the human



Figures 5. Susceptibility of MHC-AF to treatment with proteolytic enzymes. MHC-AF preparations were treated with trypsin or chymotrypsin enzymes coupled to sepharose beads for 120 min, at 37°C. Enzyme coupled beads were removed by centrifugation and the MHC-AF preparations were assayed for biological activity as described in legend to figure 2.

systems (Saxena *et al* 1992, 1996). The factor was initially identified by its property of inducing resistance to NK cell mediated lysis, in tumour cell lines (Saxena *et al* 1988). For this reason, the factor was called NK-RIF (NK-resistance inducing factor). Further studies indicated that the NK- RIF preparations were potent activators of expression of class I MHC antigens on tumour cells (Saxena *et al* 1988,1989). Since we now routinely use the MHC activation as a basic bioassay for this activity, the factor is now referred to as MHC-AF (MHC I activating factor) (Saxena *et al* 1996). We have elsewhere provided detailed accounts of properties of MHC-AF, its purification from different sources and its characterization (Saxena 1987; Saxena *et al* 1988,1996).

Since MHC-AF appears to be a hitherto unknown cytokine, it is of great interest to isolate this cytokine and determine its amino acid sequence. Attaining this objective has however posed considerable difficult. Cytokines are released in a very low concentration in the culture supernatants, ranging from few pg to few ng/ml. The culture medium in which HPBLs are cultured contains FCS which has a very high concentration of protein (about 6Omg/ml). RPMI-1640 supplemented with 5% FCS has 3 mg of protein per ml. Hence to purify a few ng of protein from Con-A supernatant

containing such high amount of protein is almost impossible. To tackle this problem we have tried a variety of culture media which are either low in protein contents or are altogether serum free. Before such media are used for generating large amounts of MHC-AF, it is crucial to select the most suitable low protein culture medium and demonstrate that MHC-AF is indeed produced in such medium. This has been the basic aim of the present study. We first attempted to compare the mitogen induced proliferative response in several test media. Results of these experiments indicate that the proliferative response of HPBls in different test media was similar. Of the culture media examined, DCCM-2 had minimum amount of protein, only 126 mg/ml, and even these proteins are defined (BSA 100 µg/ml; transferrin 25 µg/ml and insulin 1 µg/ml). Using such a culture medium for the generation of MHC-AF, ofers several advantages. Firstly, the overall amount of irrelevant proteins in crude supernatants is reduced almost 25-fold (as compared to culture medium containing 5% FCS), making it easy to process larger batches of culture supernatants. Moreover, since the culture medium proteins are well defined, it is easy to remove these by devising suitable strategies. This is very difficult in case of FCS proteins which represent an extremely complex mixture of a large number of proteins.

Our further experiments indicated that MHC-AF was secreted by activated HPBLs in DCCM-2. Partial characterization of MHC-AF secreted in DCCM-2 medium indicated that the activity is very similar to that isolated from FCS containing culture media (Saxena *et al* 1996). Thus both activities, are resistant to pH 2.0 treatment, have a molecular weight above 30 kDa. Efects of heat and proteolytic enzyme treatment on the activity of human MHC-AF had not been reported before, and the present studies indicate that human MHC-AF is susceptible to both treatments.

A major hurdle in the purification of human MHC-AF has been the presence of overwhelming amount of a large number of irrelevant FCS proteins in MHC-AF preparations. Standardization of use of serum free medium for the generation of human MHC-AF is expected to simplify the purification of this activity to homogeneity.

Acknowledgements

This work was supported by the Department of Biotechnology, New Delhi, grant to RKS. AR and NP acknowledge receipt of fellowships from the University Grants Commission, New Delhi.

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