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Inhibition of the presentation of dengue virus antigen by macrophages to B cells by serine-protease inhibitors

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Summary. It has been shown that macrophages (Mϕ) process dengue type 2 virus (DV) antigen and present it to B cells leading to their clonal expansion as shown by DV-specific IgM antibody plaque-forming cell (PFC) count in spleen. The present study was undertaken to find out the nature of enzymes responsible for the processing of DV antigen in Mϕ. DV-pulsed Mϕ were treated with seven different protease inhibitors and then assayed for antigen presentation to B cells. It was observed that maximum inhibition occurred by treatment of Mϕ with PMSF, a serine-protease inhibitor. The effect of PMSF was dose dependent and was abolished by using predigested antigen. PMSF inhibited presentation of DV and sheep RBC antigens but had no effect on presentation of bovine serum albumin which does not require processing. The results thus identify the serine group of proteases as the main enzymes involved in processing the DV antigen in Mϕ.

Keywords: Dengue virus, macrophage, serine-protease-inhibitors, antigen presentation.

Macrophages (Mϕ) ingest an antigen by endocytosis and degrade it by proteolytic enzymes to make it suitable for presentation to T and B cells for generating appropriate immune response. Intra-lysosomal proteolytic degradation could be a key event in the processing of large protein antigen by Mϕ (reviewed by Unanue *et al*, 1984; Mills 1986; Chaturvedi *et al*, 1987). Small peptide antigens and large protein antigens that have been fragmented by proteolytic enzymes can be presented by metabolically inactivated Mϕ, suggesting that small antigens do not require processing (Shimonkevitz *et al*, 1983; Allen & Unanue 1984). It has been documented that lysosomes are involved in the degradation of both exogenous (DeDuve & Wattiaux 1966) and endogenous proteins (Shaw & Dean 1980). The lysosomotropic drugs, such as chloroquine and ammonium chloride which inhibit the degradation of protein antigen in Mϕ, have a profound inhibitory effect on their capacity to present such antigens (Buus & Werdelin 1984; Rizvi *et al*, 1989).

We have observed that the presence of live Mϕ is obligatory for the presentation of dengue type 2 virus (DV) antigen to B cells leading to their clonal expansion. Heat-killed or glutaraldehyde-fixed Mϕ did not present native DV antigen but could present trypsin-digested antigen. The presentation of DV

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antigen is also inhibited by treatment of Mϕ with lysosomotropic drugs like chloroquine or ammonium chloride. Further, when Mϕ are pulsed with DV and Coxsackie B_4 virus antigens simultaneously, the two antigens compete at the level of antigen processing. These findings indicated that processing of DV antigen is essential for presentation to B cells (Rizvi *et al*, 1987; 1989; 1990).

Proteins are digested by various enzymes, the proteases, which act at different sites of the protein molecule. According to their properties the proteases have been divided into four groups, namely serine, cysteine, aspartic and metallo-proteases (Barrett 1980). The effect of a protease can be inhibited by a protease inhibitor. Thus, the role of a particular protease in a process can be roughly identified by the protease-inhibitor which blocks the process. In the present study we have tried to make use of this principle to identify the nature of the protease involved in the processing of DV antigen in Mϕ. The findings indicate a role for serine-proteases in the processing of DV antigen.

Materials and methods

Mice

Experiments were carried out on 3–4-months-old inbred Swiss albino mice maintained in the Departmental breeding house.

Antigens

Dengue type 2 virus (DV, P23085 strain) purified from the homogenate of infected mouse brain by the technique of Stollar (1969), was used in doses of 1000 LD_{50} (Chaturvedi *et al*, 1977). These preparations were assayed for infectivity by i.c. inoculation in infant mice. Sucrose-acetone-extracted freeze-dried DV antigen was kindly provided by the Director, National Institute of Virology, Pune was used in doses of 1 mg/ml. Sheep red blood cells (SRBC) were used as an antigen in some experiments in a dose of 1×10^{10} cells/ml. SRBC were collected in Alsever's solution and stored at 4°C. Before use the cells were washed with phosphate buffered saline (PBS) pH 7.2 and the count was adjusted. Bovine serum albumin (BSA, Sigma Chemical Co., St Louis MO, USA) was used in dose of 1 mg/ml.

Chemicals

Phenylmethanesulphonylfluoride (PMSF) was dissolved in methanol to obtain a 50 mM stock solution. Tosylphenylmethylchloromethyl ketone (TPCK) and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were dissolved in dimethyl sulphoxide (DMSO) in concentration of 100 μg/ml and were diluted to final concentration in the medium. Aprotinin (1 mg/ml), pepstatin A (50 μg/ml), iodoacetamide (50 μg/ml) and leupeptin (50 μg/ml) were used. All chemicals were from Sigma Chemical Company, St Louis, MO, USA. Among these chemicals, PMSF, aprotinin, TLCK and TPCK are mainly inhibitors of serine proteases (EC 3.4.21); leupeptin and iodoacetamide are inhibitors of cysteine proteases (EC 3.4.22); and pepstatin A inhibits the proteases of the aspartic (EC 3.4.23) group (Barrett 1980).

Spleen cells

Normal mouse spleens were teased out gently in chilled Eagle's minimum essential medium (MEM) containing 10% foetal calf serum (FCS, Armour Pharmaceutical Co., UK) and viable cells were counted using trypan blue dye exclusion test (Chaturvedi *et al*, 1978). The cell cultures were maintained in 5-cm glass Petri dishes for 4 days in MEM-HEPES containing 10% FCS, 5×10^{-5} M 2-mercaptoethanol and antibiotics.

Preparation of macrophage monolayer

Cells collected by lavage of the peritoneal cavity of mice were layered in 5-cm glass Petri dishes and incubated for 2 h at 37°C in presence of 5% CO_2. Non-adherent cells were

removed by washing thrice with Hank's balanced salt solution (HBSS). The glass-adherent cells contained more than 95% phagocytic cells as shown by ingestion of latex particles (Chaturvedi *et al*, 1982) and were considered macrophages (Mϕ) and were used as antigen presenting cells.

Pulsing of Mϕ with antigens

Mϕ were pulsed with DV, SRBC or BSA *in vitro* by incubating them with the antigens at 37°C for 2 h. Antigen exposures were terminated by washing the cells three times with HBSS to remove unbound antigen. The cells were then fixed with 0·5% glutaraldehyde (Koch Light Laboratories Ltd, Colnbrook, Bucks, UK) for 10 min at room temperature (Shimonkevitz *et al*, 1983) and the reaction was stopped by addition of 3 ml of 0.15 M glycine buffer. The fixed Mϕ were washed thrice and assayed for antigen presentation. In some experiments the antigens were digested by addition of 1% trypsin (Difco-250, Difco Laboratories, Detroit Michigan, USA) to protein suspended in 0.1 M ammonium bicarbonate pH 8.2. After incubation for 1 h at 37°C another identical aliquot of trypsin was added and the reaction was allowed to proceed for another hour. The sample was then centrifuged cold at 3000 *g* and the supernatant was used as digested antigen for pulsing Mϕ.

Antigen presentation assay

The antigen presentation function of Mϕ was assayed by counting the antigen-specific IgM antibody plaque-forming cells (PFC) by the localized haemolysis in gel technique of Jerne and Nordin (1963) as described elsewhere (Tandon & Chaturvedi 1977; Chaturvedi *et al*, 1977). In the in-vitro assay, 30×10^6 spleen cells were layered on the antigen-pulsed Mϕ monolayers (1×10^6 cells) and cultured for 4 days in glass Petri dishes and the PFC were then counted (Shukla & Chaturvedi 1981). For the background value the spleen cells were similarly cultured on normal uninfected Mϕ and the PFC were counted on the 4th day.

For in-vivo assay, normal mice were inoculated i.p. with 1×10^6 antigen-pulsed Mϕ and the antigen-specific IgM PFC were counted in the spleen cells on day 7 (Tandon & Chaturvedi 1977). Normal uninfected Mϕ were included for the background values. The mean data from triplicate experiments have been presented after deducting background PFC and have been analysed using Student's *t*-test for *P*-value.

Results

Treatment of Mϕ with different protease inhibitors

In this experiment an effort was made to investigate the effect of seven protease inhibitors, belonging to different groups, on the antigen processing by Mϕ. The Mϕ cell sheets were treated simultaneously with DV and different protease inhibitors for 2 h at 37°C in 5% CO_2 atmosphere. The unadsorbed antigen was removed by washing with HBSS. For controls, Mϕ pulsed with DV

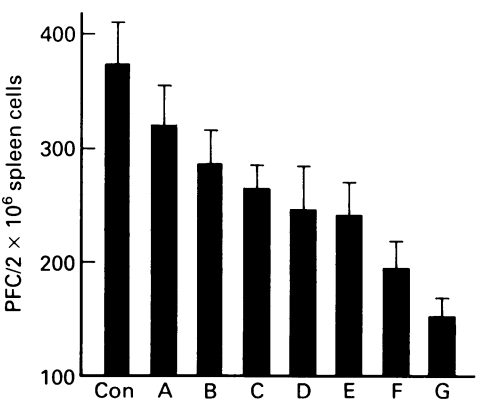

Fig. 1. Effect of treatment of Mϕ with protease inhibitors on presentation of DV-antigen to B cells. Con, untreated control; A, pepstatin-A; B, iodoacetamide; C, leupeptin; D, tosylphenylmethylchloromethyl ketone; E, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; F, aprotinin; G, phenylmethane sulphonyl fluoride.

were not treated with protease inhibitor. Antigen processing was terminated by fixation with glutaraldehyde for 10 min at room temperature. The Mϕ cell sheet was then washed thrice with glycine–HBSS buffer and the cells were then scraped off, counted, and assayed for their capacity to present the antigen. The data presented in Fig. 1 show that presentation of DV was significantly inhibited ($P<0.01$) by Mϕ treated with trypsin-like inhibitors, for example PMSF, TLCK and aprotinin. PMSF had the most striking inhibitory effect on the presentation of DV (63%) so further experiments were carried out with it.

Dose-dependent effect of PMSF

The above experiment was repeated using graded doses of PMSF (10–100 mM) for treatment of DV-pulsed Mϕ. The cell sheet was thoroughly washed, fixed with glutaraldehyde and assayed for antigen presentation. The findings presented in Fig. 2 show a dose-dependent inhibition of antigen presentation. With a 10-mM concentration of PMSF the inhibition was 38% while that with 100 mM drug was 74%.

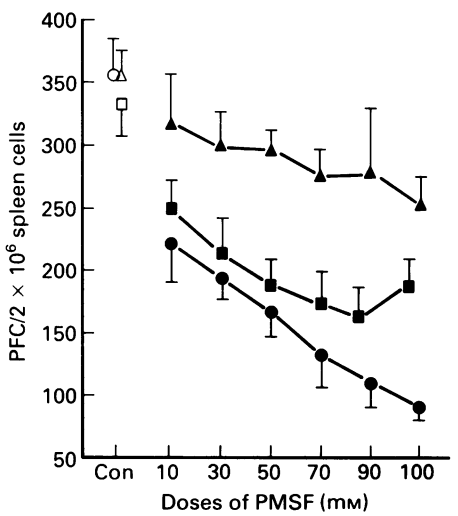

Fig. 2. Effect of treatment of Mϕ with PMSF on presentation of ●, undigested or ▲, predigested DV-antigen or ■, SRBC to B cells. Con, antigen presentation by untreated Mϕ pulsed with ○, undigested or △, predigested DV antigen or □, SRBC.

PMSF does not inhibit the presentation of predigested antigen

Experiments were conducted in which Mϕ pulsed with the predigested antigen were treated with the inhibitor to obtain evidence whether the effect of PMSF was on antigen processing. A set of Mϕ monolayers were pulsed with DV antigen predigested with trypsin followed by treatment with various (10–100 mM) doses of PMSF. The results presented in Fig. 2 show that the presentation of the predigested DV by Mϕ was inhibited to the extent of 11–28% with different doses of PMSF.

The effect of PMSF on presentation of DV and SRBC

In this experiment the selectivity of PMSF on the processing of two heterologous antigens, DV and SRBC, has been investigated. One set of Mϕ-monolayers was pulsed with DV and the other with SRBC. Both the sets of Mϕ monolayers were treated with graded doses of PMSF. The cells were allowed to process the antigen for 2 h at 37°C. The unbound antigen was removed by washing thrice with HBSS. Antigen processing was terminated by glutaraldehyde treatment. Fixation was stopped by washing the cell sheet with glycine–HBSS buffer. The cells were then assayed *in vivo* for antigen presentation by counting DV or SRBC-specific PFC. For control values, Mϕ pulsed with either of the antigens alone were included. The data presented in Fig. 2 show that presentation of the SRBC antigen was inhibited from 26 to 41% by treating the Mϕ with various doses of PMSF. The inhibition of DV-antigen presentation was much more, being 38–74%. The difference in the inhibition of the two antigens was insignificant ($P>0.05$).

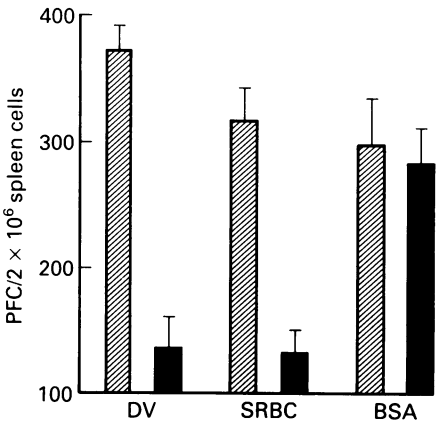

Fig. 3. Effect of treatment of Mϕ with PMSF on presentation of the antigen to B cells which does not require processing. ▨, untreated control Mϕ; ■, Mϕ treated with PMSF, pulsed with the antigens DV, SRBC or BSA.

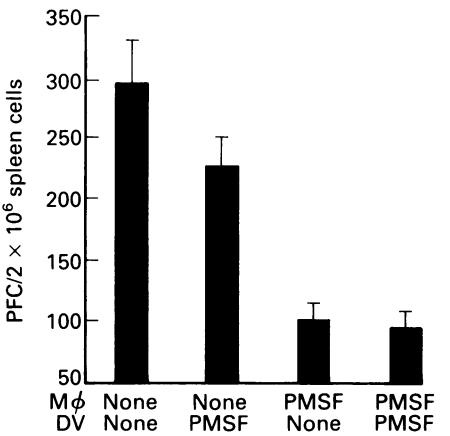

Fig. 4. Effect of treatment of Mϕ and/or DV antigen with PMSF on presentation of DV antigen to B cells.

PMSF inhibits presentation of those antigens which need processing

In this experiment the effect of PMSF on presentation of antigens that require processing was compared with those which do not require it. To study this we have taken DV and SRBC which need processing and bovine serum albumin (BSA) as an antigen which does not require processing. One set of Mϕ monolayer was treated with sucrose-acetone-extracted DV antigen, the second set was treated with SRBC, and the third set was treated with 1 mg/ml BSA. All sets received a 50-mM dose of PMSF. The antigens were allowed to adsorb for 2 h at 37°C and then unbound antigen was removed by washing thrice with HBSS. The pulsed Mϕ were then assayed *in vivo* for antigen presentation. The data presented in Fig. 3 show that PMSF inhibited the presentation of DV and SRBC. On the other hand, the presentation of BSA was similar in the two sets, one having PMSF and the other without it. This indicates that the effect of PMSF is specifically with respect to antigen processing.

Effect of treatment of Mϕ and DV antigen with protease inhibitor

This experiment was designed to study the effect of pretreatment of DV antigen with PMSF. Pure dengue antigen suspension was prepared and 1 ml of it was treated for 2 h at 37°C with a 50-mM PMSF dose. Similarly, normal mouse Mϕ sheet was prepared and treated with 50 mM PMSF for 2 h at 37°C. Experiments were made using different combinations of untreated or treated DV antigen and Mϕ as shown in Fig. 4. The antigen was allowed to adsorb on macrophage sheet at 37°C for 2 h. The unadsorbed antigen was removed by washing thrice with HBSS. The antigen-pulsed Mϕ sheet was fixed by glutaraldehyde treatment and the cells were then inoculated into normal mice for assay of antigen presentation.

The results presented in Fig. 4 show that pretreatment of DV with protease inhibitor *in vitro* had insignificant effect on the PFC counts (227 ± 26 as compared to control 294 ± 35). But the treatment of Mϕ with protease inhibitor prior to antigen pulsing showed maximum reduction in PFC count. The count with untreated Mϕ was 294 ± 35 while PMSF-treated Mϕ produced 101 ± 16 PFC. Similarly, when both Mϕ and DV were treated with the inhibitor the counts declined to 95 ± 17.

Discussion

The findings of the present study demonstrate that presentation of DV Antigen by Mϕ to B cells is inhibited by serine-protease inhibitors for example PMSF. This conclusion has been drawn by using a battery of seven protease inhibitors belonging to the three different groups, namely serine, cysteine and aspartic protease inhibitors.

The steps involved in the presentation of antigen by Mϕ include binding, uptake, ingestion, processing and expression of the antigen at the cell surface in appropriate immunogenic form (Unanue *et al*, 1984). Endocytosis of an antigen is followed by fusion of endosome with a lysosome, denaturation, and proteolytic digestion in an acidic environment. Non-lysosomal pathways of proteolysis have also been demonstrated. Both the routes of proteolytic digestion of antigen can be inhibited by lysosomotropic drugs like chloroquine or ammonium chloride (reviewed by Mills 1986). We have demonstrated earlier that Mϕ take up DV antigen, process it, and then present it to B cells leading to their clonal expansion (Rizvi *et al*, 1987). Mϕ play an obligatory role in the presentation of DV antigen to B cells because DV antigen must be in a processed form for the B cells to recognize it. Thus the presentation of DV antigen to the B cells was effectively inhibited by pretreatment of the Mϕ with the two lysosomotropic drugs; heat-killed or glutaraldehyde-fixed Mϕ do not present DV antigen; depletion of Mϕ abrogates immune response; and the need of Mϕ is abolished by predigestion of DV antigen with trypsin (Rizvi *et al*, 1989). The findings described here further show that PMSF, a serine protease inhibitor, inhibited presentation of DV antigen in a dose dependent manner. The inhibitory effect was abolished when the antigen was predigested. At the same time, presentation of the antigen (e.g. BSA), which did not require processing, was not inhibited by PMSF.

Further support for this finding has recently been obtained which shows that the antigenic competition between the DV antigen and a heterologous antigen (Coxsackie B_4 virus antigen) for presentation to B cells is abolished by digesting the DV antigen with trypsin which is a serine protease and is able to digest DV antigen into the immunogenic form *in vitro* (Rizvi *et al*, 1990). Thus the findings of the present study support and extend our results reported earlier, that DV antigen must be processed by Mϕ by a trypsin-like protease.

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