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Characterization of the dengue virus-induced helper cytokine

Pratibha Chaturvedi, Rinee Mukherjee, U.C. Chaturvedi and Asha Mathur
Postgraduate Department of Microbiology, KG Medical College, Lucknow, India

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Summary. Dengue type 2 virus (DV) induces a subpopulation of T lymphocytes of mouse spleen to secrete a soluble helper cytokine (HF) which enhances the DV-specific IgM antibody plaque forming cells (PFC). The present study undertaken to purify and characterize HF shows that it can be purified by low pressure liquid chromatography (LPLC) using Sephacryl S-200 column. HF consisted of two subunits, having a M_r of 65–68 kDa on SDS-PAGE, and both had similar activity. The isoelectric point of HF was 6.5. HF-specific antisera (HFAS) raised in mice neutralized the activity of HF in mice, reacted with it in a Western blot assay, and bound HF in an immunosorbent column. HF bound to DV-antigen in an immunosorbent column and enhanced only the DV-specific PFC. HF had no effect on PFC against heterologous antigens such as Japanese encephalitis virus, Coxsackie B_4 virus or sheep red blood cells. HF generated in mice of H-2^k haplotype, enhanced DV-specific PFC in the same strain of mice but had no effect on that in the H-2^d or H-2^q haplotype strains of mice. Thus, DV-induced HF with a M_r of 65–68 kDa, antigen-specificity and genetic-restriction differs from most of the similarly acting cytokines but appears similar to the cell-free form of T cell receptor $\alpha\beta$ dimer.

Keywords: helper cytokine, dengue virus, antigen-specific, genetic restriction

Antibody production in response to thymus dependent antigens results from a complex series of cellular interactions in which antibody forming cell precursors (B-lymphocytes) proliferate and differentiate to synthesize specific antibody. The regulation of this response involves cellular and humoral interactions in which thymus derived lymphocytes (T-cells) act as helpers or suppressors (reviewed by Wagner *et al.* 1982). Feldmann and Basten (1972) were the first to report that the helper signal can be transmitted through a secretory soluble product of the cells, the helper factor. This factor has been described in many systems using synthetic antigens (Howie & Feldmann 1977; Mozes & Haimovich 1979; Feldmann & Kontiainen 1981; Wecker & Schimpl 1981; Zanders *et al.* 1980).

Dengue type 2 virus (DV) infection in mice sequentially induces a T helper cell (TH) population followed by three distinct sets of T suppressor cells (Chaturvedi 1984; Chaturvedi *et al.* 1987; Shukla & Chaturvedi 1984). DV-induced TH are antigen-specific and express Thy1.2$^+$, Ly1$^+$23$^-$, I-J$^-$ and I-A$^+$ surface phenotype (Chaturvedi *et al.* 1987; unpublished data). The generation of

Correspondence: Professor U.C. Chaturvedi, Department of Microbiology, KG Medical College, Lucknow 226 003, India.

DV-induced TH is regulated by the suppressor and cytotoxic pathways induced by the virus (Chaturvedi *et al.* 1985; Khanna *et al.* 1988, 1989). The DV-induced TH produce a soluble helper cytokine (HF) which is heat, acid and alkaline pH-labile (Pahwa *et al.* 1988).

Mouse TH clones have now been divided into two distinct types, TH1 and TH2, but in normal mouse T-cell populations their origin is not clear. It is also known that TH1 secrete interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor (TNF) while TH2 secrete IL-4 and IL-5 (reviewed by Mosmann & Coffman 1989). The functional distinction between the TH cell subsets appears to lie in the soluble factors synthesized by them, because the contact-dependent signals of activated TH cells from distinct subsets are similar (reviewed by Noelle & Snow 1990). The above mentioned soluble factors secreted by the TH-subsets are well characterized. The present study was, therefore, undertaken to purify and characterize the DV-induced HF.

Materials and methods

Animals

The adult inbred Swiss albino mice used were aged 3–4 months and were obtained from the colony maintained in this Department. Mice of BALB/c and DBA strain were purchased from the Central Drug Research Institute, Lucknow.

Virus

Dengue type 2 virus (DV), strain P23085 was used in the form of infected adult mouse brain suspension (Chaturvedi *et al.* 1977).

Preparation of HF

DV-specific helper T cells were generated in the spleen of mice by the technique described earlier (Chaturvedi *et al.* 1985, 1987). Briefly, mice injected with 100 LD_{50} of DV intravenously were killed on day 4 and the spleens collected. A single-cell suspension of the spleen was prepared in phosphate-buffered saline pH 7.2 (PBS) and cultured for 24 h at 37°C in presence of 5% CO_2. The cell-free supernatant was obtained by centrifugation at 4°C for 10 min at 3000 *g*. The helper cytokine (HF) was purified by Pharmacia low pressure liquid chromatography (LPLC) using Sephacryl S-200 gel packed in 650 × 16 mm column with a bed height of 450 mm. Elution was performed with PBS at 40 cm^3/h and the elution fluid was monitored at 280 nm with a UV-cord. The protein peaks obtained were collected separately and freeze dried or vacuum dried in a Speed Vac (Savant Instruments Inc., USA) and stored at −70°C. It was reconstituted with distilled water and the protein content was estimated by the method of Lowry *et al.* (1951). For control, a similar preparation from normal mouse spleen cells (NF) was used in each experiment. The helper activity of each peak was assayed.

Preparation of HF-specific antisera (HFAS)

Antisera against purified HF (Peak 4 of Fig. 1) was prepared in mice by giving two intraperitoneal injections of 5 μg HF emulsified in complete Freund's adjuvant at 15 days interval. Mice were bled on the 30th day and the sera were separated and stored at −20°C. Similarly, antisera were prepared against the peak 1 (HFAS-1) and peak 2 (HFAS-2) of Fig. 3, obtained by rerun of purified HF on LPLC.

Preparation of immunosorbent columns

Immunosorbent columns of HFAS or DV-antigen coupled with cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals) were prepared (Fuchs & Sela 1986). Preparations of HF were adsorbed on the column and the effluent and the eluate thus obtained were assayed for helper activity. For control, columns prepared with

normal mouse sera (NS) or normal mouse brain extract (NB) were used.

Polyacrylamide gel electrophoresis (PAGE)

HF preparations were electrophoresed on 12% polyacrylamide native gels (PAGE) or sodium dodecyl sulphate (SDS)-PAGE (Lammeli 1970) at constant current of 30mA in a vertical slab gel system. Gels were stained with silver nitrate (Merril *et al.* 1981) or with Coomassie Brilliant Blue R250 stain. Some of the gels were run with molecular weight markers (Weber & Osborn 1969).

Isoelectric focusing

Isoelectric point (PI) of purified HF was determined using polyacrylamide gel containing Ampholine (pH 3.5–10; Pharmacia) in a Multiphore II electrophoresis unit and electrofocusing electrodes (Pharmacia, Sweden). The anodic solution was 0·04 M orthophosphoric acid and 1M sodium hydroxide was used as cathodic solution. The gels were prefocused for 15 min at 15W constant power. Focusing was done by running the gels at 15W for 15 min and then at 20W for 3 h. Markers of pH range 3.5–9.5 (Sigma Chemical Co., USA) were run along with the sample. The gels were fixed in a fixative containing trichloroacetic acid and sulphosalicylic acid and stained with Coomassie R250 stain. PI of HF was interpolated from the positions of the markers.

Immunoblotting

HF or its purified fractions and the NF proteins (for control) were resolved on 12% native or SDS-PAGE gels and then electroblotted onto nitrocellulose paper. It was blocked with 3% bovine serum albumin prepared in blocking buffer, containing 0.1 M Tris HCl pH 7.5 and 0.05% Triton X-100. The blots were incubated with appropriately diluted HFAS at room temperature (25°C) on a rocking table. After three washings it was incubated with anti-mouse IgG linked to horseradish peroxidase for 2.5 h at room temperature. After extensive washing, the blots were developed using diaminobenzidine and hydrogen peroxide. For control, the blots were treated with anti-DV-antisera (Chaturvedi *et al.* 1991b) or normal mouse sera (NS) in place of HFAS.

Assay of helper activity

The helper activity was assayed by counting DV-specific IgM antibody plaque forming cells (PFC) in the spleens of mice as described elsewhere (Chaturvedi *et al.* 1985, 1987). Cyclophosphamide (CY) eliminates the precursors of T helper cells (Shand & Liew 1980) so the mice were pretreated with CY (Endoxan ASTA, Khandelwal Industries, Bombay) in doses of 200 mg/kg body weight to remove endogenous T helper cells. This was followed by 10^3 LD_{50} of DV i.p. after 24 h, and 72 h later with HF in doses of 0.2 ml i.v. DV-specific PFC were counted after 96 h of inoculation of DV. DV-specific IgM (direct) PFC were counted by the localized haemolysis in gel technique of Jerne and Nordin (1963) as described by Tandon and Chaturvedi (1977). For background values, in each experiment a set of mice were included which were treated with CY and given PBS in place of HF. The data have been presented as mean ± s.d. from 10–15 mice in each group, after deducting the background values. The increase in PFC count by treatment with HF is presented as per cent help which was calculated as follows:

$$\% \text{ Help} = 100 \times \frac{\text{Number of PFC in DV primed mice given HF} - \text{background value}}{\text{Number of PFC in DV primed mice} - \text{background value}} - 100$$

In some of the experiments effect of HF on PFC against Japanese encephalitis virus (JEV; Mathur *et al.* 1981), coxsackie B_4 virus (CoxB; Chaturvedi *et al.* 1978) and sheep red blood cells (SRBC; Chaturvedi *et al.* 1981) were studied as described. The data have been analysed using Student's *t* test for *P*

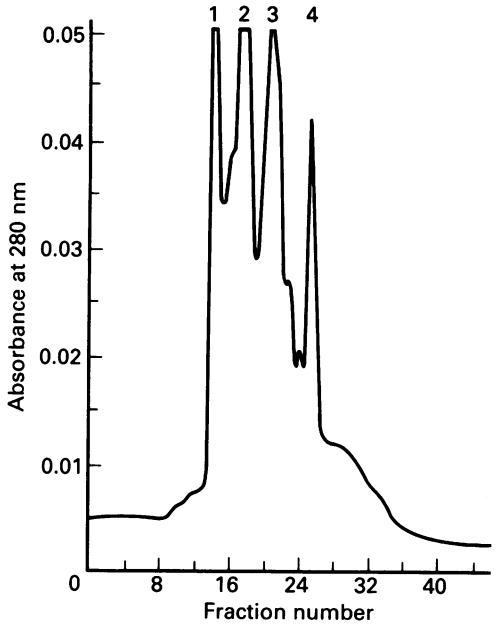

Fig. 1. Purification of crude HF on low pressure liquid chromatography (LPLC) using Sephacryl S-200 column. The fractions were monitored for protein at 280 nm with a UV-cord.

value. A P value of less than 0.05 was considered significant.

Results

Purification of HF

The elution profile of crude HF purified on LPLC has been presented in Fig. 1. Four major protein peaks eluted were assayed for helper activity. The findings presented in Fig. 2 show that antigen-specific PFC count in DV-primed mice was 451 ± 45 per 2×10^6 spleen cells, which increased to 935 ± 83 when untreated HF was given, the help being 107%. Proteins obtained from peaks 1, 2 or 3 had negligible effect on the PFC count. On the other hand, an increase of 90% was seen when mice were treated with the peak 4. A splitting of peak 4 into two, a smaller and a larger peak, was noted in some of the runs on LPLC. Proteins from both these peaks had similar enhancing effect on the PFC count.

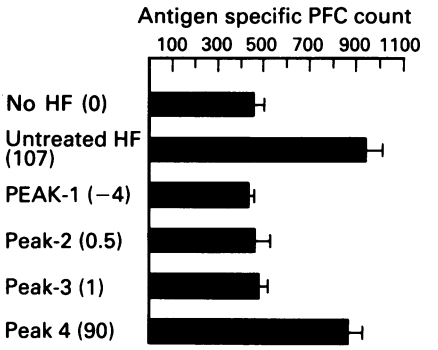

Fig. 2. Helper activity of the protein peaks eluted from the LPLC. Each peak was freeze dried, reconstituted and assayed for helper activity. The percentage help (values in parentheses) was calculated by the values obtained in DV-primed mice which were not given HF. The preparations obtained from normal mouse spleen cells had no effect on the PFC count so the data is not presented.

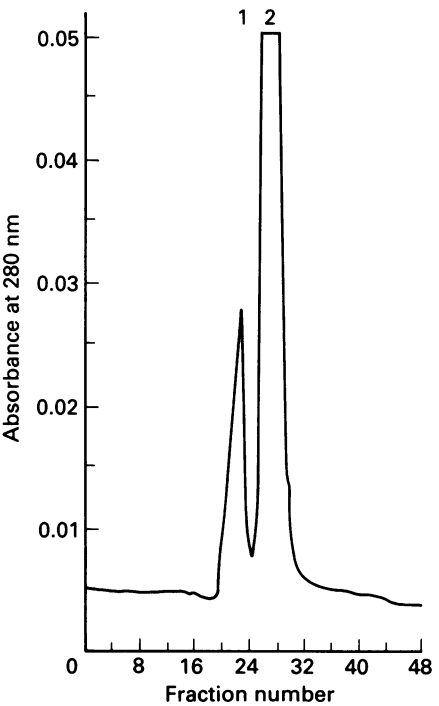

Fig. 3. Elution profile of purified HF on LPLC. The LPLC purified HF (peak 4 of Fig. 1) was rechromatographed on the same LPLC column. The two peaks eluted were freeze dried and assayed; both the peaks had similar helper activity.

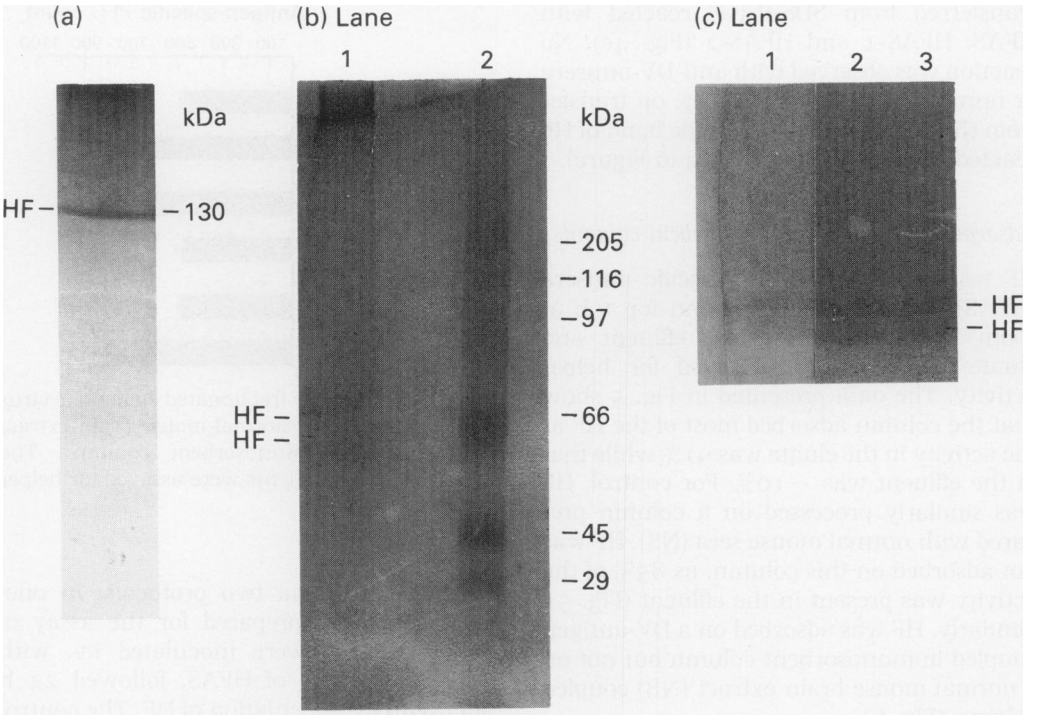

Fig. 4. Polyacrylamide gel electrophoresis of purified HF on a, native-PAGE and b, SDS-PAGE with M_r markers. c, Western blot analysis was done by transfer of protein from SDS-PAGE using HFAS (Lane 1), HFAS-1 (Lane 2) or HFAS-2 (Lane 3).

Similar splitting was observed when the purified peak 4 was freeze dried and re-chromatographed on LPLC (Fig. 3); both the peaks had similar activity. If the crude HF was frozen it aggregated and it was difficult to run on LPLC.

Polyacrylamide gel electrophoresis (PAGE)

LPLC-purified HF (peak 4 of Fig. 1) was dried in a Speed Vac and was electrophoresed in native PAGE and the position of HF was identified by cutting one lane of the gel and quickly staining it; the stained lane was aligned by the side of the main gel and portions were cut along the position of the protein band. The protein eluted from the gel was found to have the helper activity. A comparison with various molecular weight markers indicated that HF had a M_r of 130 kDa (Fig. 4a). The purified HF protein was denatured with 1% SDS at 90°C for 5 min under reducing conditions and then it was electrophoresed on SDS-PAGE. HF was split into two bands of smaller M_r occupying a position of 65–68 kDa on SDS-PAGE (Fig. 4b). The protein from each of the two peaks obtained on re-run on LPLC (Fig. 3) gave a single band on SDS-PAGE at 65 and 68 kDa positions.

Isoelectric focusing

The isoelectric point (PI) of HF was found to be 6.5. When the HF was run following treatment with SDS, both the bands had similar PI. The two peaks of HF separated on LPLC (Fig. 3) yielded similar results.

Immunoblotting

It was observed that both the bands of HF

transferred from SDS-PAGE reacted with HFAS, HFAS-1 and HFAS-2 (Fig. 4c). No reaction was observed with anti-DV-antisera or normal rabbit sera; similarly, on transfer from the native-PAGE, the single band of HF reacted with HFAS (not shown in Figure).

Adsorption of HF on immunosorbent columns

HF was incubated on HF-specific antisera (HFAS)-immunosorbent column for 1 h at room temperature and the effluent and eluate collected were assayed for helper activity. The data presented in Fig. 5 show that the column adsorbed most of the HF as the activity in the eluate was 91% while that in the effluent was −10%. For control, HF was similarly processed on a column prepared with normal mouse sera (NS). HF was not adsorbed on this column, as 85% of the activity was present in the effluent (Fig. 5). Similarly, HF was adsorbed on a DV-antigen coupled immunosorbent column but not on a normal mouse brain extract (NB) coupled column (Fig. 6).

Inhibition of helper activity by specific antibody

The effect of HFAS on the helper activity of

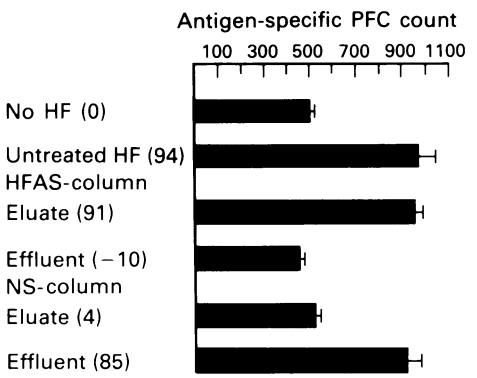

Fig. 5. Activity of HF fractionated on anti-HF-antisera (HFAS) coupled immunosorbent columns. Normal mouse sera (NS) coupled immunosorbent columns were used for control. The eluates and the effluents were assayed for helper activity. Percentage help values are given in parentheses.

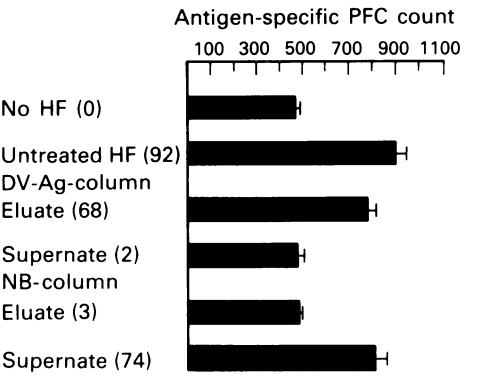

Fig. 6. Activity of HF fractionated on dengue virus antigen (DV-Ag) or normal mouse brain extract (NB) coupled immunosorbent columns. The eluates and the effluents were assayed for helper activity.

HF was tested via two protocols. In one, groups of mice prepared for the assay of helper activity were inoculated i.v. with various dilutions of HFAS, followed 24 h later with i.v. inoculation of HF. The control mice received either normal mouse sera (NS) or PBS in place of HFAS. The findings presented in Fig. 7 show a dose-dependent inhibition of the helper activity by treatment with HFAS. A maximum inhibition of 97% of the helper activity occurred by treatment with neat HFAS while that with 10^{-5} diluted HFAS was 19%. In the second protocol, HF was mixed with equal volumes of HFAS at 37°C for 1 h followed by centrifugation at 6000 *g* for 10 min. The supernatants were assayed for helper activity. The above controls were included in each experiment. By this protocol, the neat HFAS could inhibit only 42% of the helper activity.

Antigen specificity of HF

The antigen specificity of HF was investigated by its effect on various heterologous antigen-specific PFC, like JEV, CoxB or SRBC. The activity of HF was assayed in groups of mice primed with either of these antigens and compared with that in DV primed mice. The findings presented in Table 1 show that treatment of mice with HF had no effect on

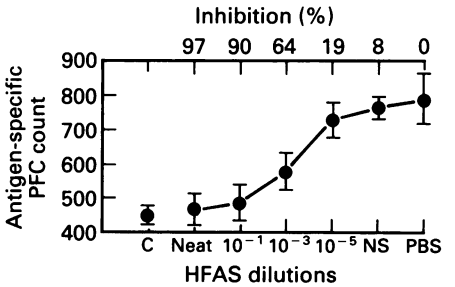

Fig. 7. Inhibition of helper activity by pretreatment of mice with HF-specific antisera (HFAS). DV-primed mice were given various dilutions of HFAS i.v. 24 h before HF and the helper activity was assayed. The percentage of help was calculated from the values in mice which were not given HF or HFAS (C) (see Materials and methods). Control mice were given normal mouse sera (NS) or the diluent (PBS). The percentage inhibition of helper activity was calculated as follows:

$$\text{Percentage inhibition} = 100 - \frac{\%\ \text{help with HF+HFAS}}{\%\ \text{help with HF+PBS}} \times 100$$

Table 1. Effect of HF on the heterologous antigen specific PFC count

Priming antigen	HF treatment	Antigen specific PFC/2 × 10^6 spleen cells	Percentage help
DV	+	807±52	97
DV	−	409±33	0
JEV	+	569±27	−5
JEV	−	600±43	0
CoxB	+	497±29	2
CoxB	−	486±48	0
SRBC	+	676±39	1
SRBC	−	668±50	0

The effect of HF on the PFC against different heterologous antigens was investigated. Mice primed with Japanese encephalitis virus (JEV), coxsackie type B_4 virus (CoxB), sheep red blood cells (SRBC) or DV were given HF (+) and IgM PFC against the specific antigen were counted. The percentage help was calculated from the counts in mice which were not given HF (−).

Table 2. Effect of HF in mouse strains of different H-2 haplotype

Name of strain	H-2 haplotype	HF treatment	No. of PFC/2 × 10^6 cells	Percentage help
Swiss	H-2^k	+	933±35	107
Swiss	H-2^k	−	456±20	0
BALB/c	H-2^d	+	445±56	4
BALB/c	H-2^d	−	426±27	0
DBA	H-2^q	+	442±43	−2
DBA	H-2^q	−	451±50	0

Activity of HF was assayed in mice of different H-2 haplotypes primed with DV, to study the genetic restriction.

the PFC against JEV, CoxB and SRBC antigens.

Genetic restriction of HF activity

The activity of HF was assayed in mice of different H-2 haplotypes (Table 2) to study the genetic restriction of its activity. For this, mice of BALB/c and DBA strains were primed with DV and the activity of HF was assayed. The findings presented in Table 2 show that HF had a negligible effect on DV-specific PFC in BALB/c and DBA mice while in Swiss albino mice the enhancement of the PFC count was 105%.

Discussion

The present study describes the purification of protein molecule (HF) obtained from the culture supernatant of DV-primed mouse spleen cells which on adoptive transfer enhances the clonal expansion of DV-specific IgM antibody forming cells. HF acted in an antigen-specific and H-2 restricted manner. It was observed that HF could be purified on LPLC as a single peak or two closely associated peaks; when eluted as a single peak, it separated into two peaks on re-chromatography. The LPLC purified HF appears to aggregate and was located at the 130 kDa

position which under reducing conditions on SDS-PAGE was split into two bands located at 65–68 kDa positions. When it was split into two peaks on LPLC, each peak gave a single band (65 or 68 kDa) on SDS-PAGE. Further, both peaks had similar helper activity and reacted equally with the antisera raised against either of the two (HFAS-1 and HFAS-2) in a Western blot test. The isoelectric point of HF was 6.5. The findings thus show that DV-specific HF molecule has at least two loosely bound moieties which are equally active. It has also been shown that both moieties of HF consist of two polypeptide chains, bound by disulphide linkage, which could be obtained in pure form by HPLC following treatment with dithiothreitol and iodoacetamide (Chaturvedi *et al.* 1991a). The M_r of HF is similar to the streptococcal antigen-specific helper factor with 70 kDa M_r (Zanders *et al.* 1980) while others are in the range 25–80 kDa (Munro *et al.* 1974; Mudawwar *et al.* 1978; Shiozawa *et al.* 1979).

HF was analysed on immunosorbent columns to determine its characteristics. It was observed that HF bound to DV-antigen and HFAS, thus showing the presence of specific combining sites on the molecules. Thus, HF appears to have the same structural features as other helper factors analysed in this manner (Feldmann & Kontiainen 1981). HF also reacted with the specific antibody in Western blot assay and its activity was abolished by pretreating the mice with HFAS. Similar findings have been described in different cytokine models *in vitro* and *in vivo* (Culbert *et al.* 1982; Ohara & Paul 1985; Khanna *et al.* 1990; Chaturvedi *et al.* 1991b).

We have shown that HF consists of two polypeptide chains bound by disulphide linkage which can be separated by treatment with reducing agents. The individual polypeptide chains bear I-A or DV-antigen determinants and have no activity individually, but the activity is restored by mixing them together (Chaturvedi *et al.* 1991a). Shiozawa *et al.* (1979) have described an alloantigen-specific helper factor which splits into two inactive units in the absence of Ca^{2+} which regain activity on mixing in the presence of Ca^{2+}.

HF was active in the Swiss strain (H-2^k) of mice in which it was produced but had no activity in BALB/c (H-2^d) and DBA (H-2^q) mice. This indicates a genetically restricted T–B interaction in the DV-model as well. This is similar to the antigen-specific and mouse strain-specific helper factors described by Shiozawa *et al.* (1977) and Leclercq *et al.* (1986). In contrast, several other helper factors are not even species specific (reviewed by Feldmann & Kontiainen 1981).

HF is produced by DV-induced TH which are antigen-specific and express Thy1.2^+, Ly1^+23^-, I-J^- and I-A^+ surface phenotype (Chaturvedi *et al.* 1987; unpublished data). TH are induced by presentation of an antigen by macrophages (Mϕ) or B cells, Mϕ being essential for particulate antigens (Chesnut *et al.* 1982). It has been suggested that antigen presentation by B cells may be a means primarily for interaction with TH and subsequent activation (Unanue *et al.* 1984). The DV antigen must be processed by Mϕ before presentation to B cells as they can not handle it by themselves; therefore, it has been suggested that both Mϕ and B cells (after receiving the antigen from Mϕ) can present the DV antigen to recruit TH (Rizvi *et al.* 1989, 1990, 1991) which in turn secrete HF to expand the B cells. Thus action of HF on B cells is at the later phase of the immune response.

The question which remains to be answered is the relationship of HF with known cytokines which stimulate B cells (reviewed by Balkwill & Burke 1989). Further, IFN-γ can also substitute for a late acting helper factor which acts synergistically with other helper factors in the stimulation of B-cell antibody response (Leibson *et al.* 1984). HF differs from these cytokines in having a higher molecular weight, antigen-specificity and genetic restriction. Guy *et al.* (1989) have described a disulphide-linked dimer, indistinguishable from the T cell surface $\alpha\beta$ heterodimer, that precipitates from cell-free supernatants of cloned T helper cells with a monoclonal antibody specific for T cell receptor (TCR) Vβ8 determinant. The affinity

purified material has antigen-specific and MHC-restricted helper activity in the generation of B cell antibody response. Guy *et al.* (1989) have suggested that this is a cell-free form of the TCR $\alpha\beta$ dimer. HF has several similarities with this product. It may be useful to know the aminoacid sequence, mRNA and cDNA of HF to establish any homology with the known cytokines and Vβ8 determinants having similar functions.

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