Immunology 1980 **41** 587

Partial characterization of murine and monkey helper factor to a streptococcal antigen

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Accepted for publication 22 May 1980

Summary. Helper factors specifically stimulating co-operative antibody responses by normal mouse spleen cells to a dinitrophenylated protein antigen from *Streptococcus mutans* (DNP-SA) were produced *in vitro* from monkey peripheral blood leucocytes and mouse spleen cells. The factors were partially characterized by gel filtration on Sephadex G-75, isoelectric focusing, treatment with heat and degradative enzymes and binding to specific immunoadsorbents. Gel filtration of both the monkey and mouse factors showed coelution with human serum albumin, suggesting a molecular weight of approximately 70,000. The isoelectric points fell within the range of 4·9–5·2 for monkey and 6·4–6·7 for the mouse helper factors. The glycoprotein nature of both factors was suggested by their lability to heat and sensitivity to pronase and neuraminidase. The factors carried a small fragment of the stimulating antigen and showed specific binding to SA but not to keyhole limpet haemocyanin (KLH). Monkey factor bound to rabbit antisera directed against the Fc portion of monkey IgM, but not to the IgG or IgA isotypes. The mouse factor contained determinants coded for by the I-A^k but not I-J^k subregion of the MHC. Both factors were absorbed by an antiserum to helper factor raised in rabbits against a KLH-specific mouse helper factor as immunogen. A corresponding antiserum to suppressor factor failed to adsorb either factor. This emphasizes the specific identities of helper and suppressor factors and suggests an evolutionary relationship between those derived from monkey and mouse leucocytes.

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0019-2805/80/1100-0587$02.00

INTRODUCTION

One of the most important causes of dental caries is colonization of the tooth surface by *Streptococcus mutans* (Fitzgerald & Keyes, 1960; Krasse, 1966). The rhesus monkey has been used as a model for studying the immune response to *Streptococcus mutans* under conditions comparable to those in man (Lehner, Challacombe & Caldwell, 1975, 1976). It has become increasingly clear however, that antibody responses to most soluble antigens are modulated by the interaction between cellular subsets derived from the bone marrow and thymus (Claman & Chaperon, 1969). Some of these interactions may be effected by soluble factors resulting in either the suppression (Tada 1975; Kontiainen & Feldmann, 1977; Germain, Theze, Kapp & Benacerraf, 1978) or enhancement of T-cell dependent antibody secretion by B cells (Feldmann, 1972; Kontiainen & Feldmann 1973; Taussig, 1974).

We wished to examine the role of cellular co-

operation in the generation of antibody responses to a protein antigen SA purified from *Steptococcus mutans* (Russell & Lehner, 1978) using the rhesus monkey as a sub-human primate model for the induction of and protection from caries (Lehner *et al.*, 1975). Previous work (Lamb, Kontiainen & Lehner, 1980) has shown that T cells separated from monkey peripheral blood and stimulated with antigen in the Marbrook–Diener culture system release a soluble factor termed helper factor. This was shown in the presence of hapten conjugated antigen to act on mouse B cells freed of T cells by treatment with anti-Thy 1 serum and complement (Lamb, J.R. unpublished results), producing a specific anti-hapten response in an antibody plaque assay. Antigen specificity was demonstrated by its non-responsiveness to the unrelated antigen KLH.

Factors were produced by nylon-wool non-adherent, anti-Thy 1 sensitive cells. As a first step towards the manipulation of immune responsiveness to an antigen which may be related to human disease, an attempt was made to characterize the primate helper factor by fractionation of helper cell culture supernatants as well as by serological and enzymatic analysis. An analogous helper factor obtained from mouse spleen cells (Lamb *et al.*, 1980) was similarly treated for comparison and the relationship between these factors and the helper factor described by Howie & Feldmann (1977) was examined.

MATERIALS AND METHODS

Animals

Three- to six-month-old B10.BR mice of both sexes were obtained from the Imperial Cancer Research Fund breeding unit and used as a source of spleen cells. Young rhesus monkeys, kept at Guy's Hospital, were bled from the femoral vein into bottles containing heparin as a source of peripheral blood leucocytes (PBL).

Antigens and antisera

Streptococcus mutans antigen was prepared from culture supernatants as described by Russell & Lehner (1978). Dinitrophenylated SA had 5 DNP groups per 100,000 mol. wt units, and trinitrophenylated (TNP) KLH had 8 per 100,000 mol. wt units. Keyhold limpet haemocyanin (KLH) was a kind gift of Dr M. Rittenberg. Antisera to mouse immunoglobulin (anti-mouse Ig) and to I subregions of the MHC (anti-Ia^k, Ia^s, $I\text{-}A^k$, $I\text{-}J^k$) were generously provided by Dr M. Feldmann. Class-specific antisera to monkey immunoglobulins (anti-monkey IgM, IgG and IgA) were purchased from Nordic Diagnostics Ltd, Maidenhead, Berks. Their specificities were checked by immunoprecipitation with corresponding antigen. These antisera were raised in rabbits, and the antisera to the Fab portion of mouse Ig in sheep. The antiserum to the streptococcal antigen was raised in rabbits following intramuscular injection of antigen in Freund's complete adjuvant. One hundred micrograms of the antigen in 1 ml adjuvant was injected into the hind legs and followed after 1 month with 100 μg in Freund's incomplete adjuvant injected subcutaneously in the back. A crude IgG fraction was obtained from the resulting immune serum by two successive precipitations with an equal volume of saturated ammonium sulphate. The resolubilized precipitate was dialysed against 0·1 M sodium bicarbonate, 0·5 M sodium chloride for 24 h. Antisera to KLH-specific helper and suppressor factors were prepared in rabbits as described in detail previously (Kontiainen & Feldmann, 1979).

Preparation and assay of factors

The production of mouse and monkey SA-specific helper cells and factors has been described in detail by Lamb *et al.* (1980). Briefly, monkey peripheral blood leucocytes or mouse spleen cells were suspended at 5×10^6 viable cells in 1 ml of tissue culture medium and were incubated in the insert of a modified Marbrook–Diener flask with 0·01 μg/ml of SA. After 4 days of culture the helper cells were harvested, washed and the number of viable cells counted. To obtain SA-specific helper factor, the cells were resuspended to 5×10^6 viable cells and cultured together with 0·1 μg of SA/ml for an additional 24 h. Supernatants harvested after 24 h are reterred to as helper factors. The assay of helper factors was in a co-operative culture system as previously described (Lamb *et al.*, 1980). As factors appear not to show species specificity, monkey factors could be assayed on mouse spleen cells. Briefly, 10^7 normal (unprimed) spleen cells were cultured for 4 days together with the optimal dilution of helper factor and 0·1 μg/ml DNP-SA. Triplicate cultures were used throughout. Anti-DNP antibody producing cells (AFCs) were assayed by the Cunningham plaque assay using DNP-Fab coated sheep erythrocytes as indicator cells (Cunningham & Szenberg, 1968). With unprimed spleen cells only IgM AFC were detected. Results are expressed as mean AFC/culture $\pm$ standard error. A thymus-independent antigen, DNP-

polycrylamide beads (DNP-PAA, kindly supplied by Dr M. Baltz) was used as a control antigen to test the reactivity of B cells. Background numbers of AFC given by unprimed spleen cells without added helper factor were determined by cultivating spleen cells either alone or with 0·1 μg/ml DNP-SA.

Gel exclusion chromatography

Helper factor from 5×10^6 SA-induced helper cells in a volume of 1 ml was applied to a 2×90 cm column of Sephadex G-75 (Pharmacia Ltd, Hounslow, Middx) pre-calibrated with 10 mg each of human serum albumin (Calbiochem, Bishops Stortford, Herts.), ovalbumin (Sigma Ltd, Poole, Dorset) and lysozyme (Worthington, Freehold, N.J.). The void volume was determined with blue dextran (Pharmacia). After elution at 20 ml/h in phosphate-buffered saline (PBS), 3 ml fractions were collected. Fractions were assumed to be at a 1:3 dilution with respect to helper activity. Selected tubes, encompassing the void volume and ovalbumin at a mol. wt of 45,000, were assayed at a $1{:}10^{-3}$ final dilution in cooperative cultures as described above. A pool from the rest of the eluate was also assayed at this dilution.

Isoelectric focusing

The micropreparative procedure of Sorg & Bloom (1973) was employed using a sucrose gradient as the anti-convective medium. Briefly, 200 μl of unfractionated factor was distributed within a 3·3 ml gradient of sucrose from 18% (w/v) to 40% (w/v) containing 100 μl of ampholines pH 3·5–10 (LKB Ltd, Bromma, Sweden). After focussing for 17 h at 500 V, fractions of approximately 200 μl were collected and diluted 1:10 in sterile water. After measurement of the pH of each fraction, selected samples were assayed for helper activity at a final dilution of $1{:}10^{-3}$.

Heat and enzyme treatment of factor

Undiluted mouse and monkey helper factors were frozen at −20° and thawed at 37° over five cycles, and then assayed at $1{:}10^{-3}$ dilution. Twenty-five microlitres of cell-free supernatants, made up to 250 μl in TC 199 medium, were heated to 37°, 60° and 80° for 0·5 h in a water bath. Samples were then frozen and assayed at $1{:}10^{-3}$ as before. Background values were determined by culturing unprimed spleen cells with DNP-SA without added factor, as well as culturing B cells alone. Enzymic digestion was performed using agarose insolubilized enzymes (Sigma), with the exception of pronase, which was coupled to CM-cellulose; pronase (0·25 μmol), neuraminidase (0·025 μmol), β-glucuronidase (0·025 μmol), and ribonuclease (0·25 units), were added to 250 μl of a 1:10 dilution of helper factor. Dilutions were made in 100 mM Tris acetate, pH 5·0, except for pronase which was suspended in 100 mM Tris chloride, 10 mM calcium chloride, pH 8·0. Reactions were initiated by the addition of 100 μl of washed enzyme suspension and continued at 37° for 0·5 h. Reactions were terminated by centrifugation in a Beckman microfuge for 1 min and freezing the enzyme-free supernatants. Samples were assayed in cooperative cultures at $1{:}10^{-3}$ dilution. Any effects due to buffer were tested for by using helper factor alone with each of the two buffers as controls.

Preparation and use of immunoadsorbents

Protein antigens, fractionated immunoglobulins and antisera were coupled to Sepharose 4B activated with cyanogen bromide (Pharmacia). The manufacturer's instructions were followed using 1 mg KLH or SA, approximately 10 mg of fractionated serum protein and 100 μl of whole antiserum per ml of beads. After inactivation of free reactive groups with 0·1 M ethanolamine pH 9·0, 0·5 ml volumes were washed once in saline (0·9% sodium chloride), once in Sorensen's buffer (glycine hydrochloride pH 2·3) and twice in saline. For the adsorption of HF 0·5 ml of $1{:}10^{-2}$ dilution of helper factor was adsorbed with 0·5 ml of beads on a rotary mixer. After 2 h adsorption at 4°, the unbound material was recovered by centrifugation at 400 r.p.m. for 3 min, on a bench centrifuge. After dilution with an equal volume of saline, samples were filtered through millipore filters and stored at −20°. After the beads had been washed four times with saline, bound material was eluted from the beads with 0·5 ml Sorensen's buffer, pH 2·3, and dialysed against saline for 24 h. After dilution with 0·5 ml of saline, the eluate was treated as above. Both fraction were assayed in cooperative cultures at concentrations of $1{:}10^{-3}$.

Statistics

Data were subjected to statistical analysis using the Student's *t* test.

RESULTS

Molecular weights of murine and monkey factors

Gel filtration of culture supernatants was used to estimate the approximate molecular weight of helper fac-

tors. The elution profiles of murine and monkey helper activities are shown in Fig. 1. Histogram points represent the mean of three assays with standard errors. Based on the co-elution of both factors with human serum albumin, the molecular weights were estimated to be about 70,000. Fractions not assayed individually were pooled so that each individual fraction was present at a dilution of $1:10^{-3}$. In these fractions, background levels of activity were obtained (data not shown). The same results were obtained in at least twelve experiments.

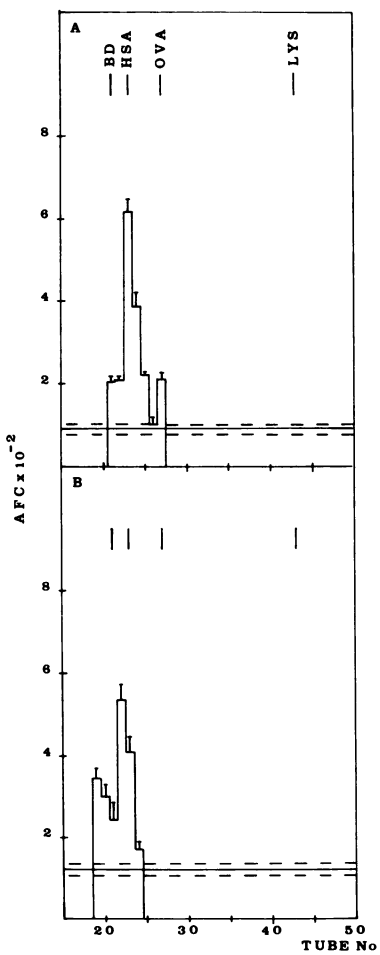

Figure 1. Sephadex G-75 chromatography of (A) mouse and (B) monkey helper factor. Each point represents the mean ± SE of triplicate assays. Background AFCs ± SE are indicated by a solid line. Molecular weight markers are: BD, blue dextran; HSA, human serum albumin; OVA, ovalbumin; and LYS, lysozyme.

Isoelectric focusing of helper factors

After isoelectric focusing, both the monkey and mouse helper activity formed a single peak, ranging in different experiments from 4·9 to 5·2 for monkey, and 6·4–6·7 for mouse factors (Fig. 2). The presence of ampholines did not appear to have an inhibitory effect on either factor since unfractionated material, when mixed with the appropriate concentration of the former did not have reduced activity relative to control factors (data not shown).

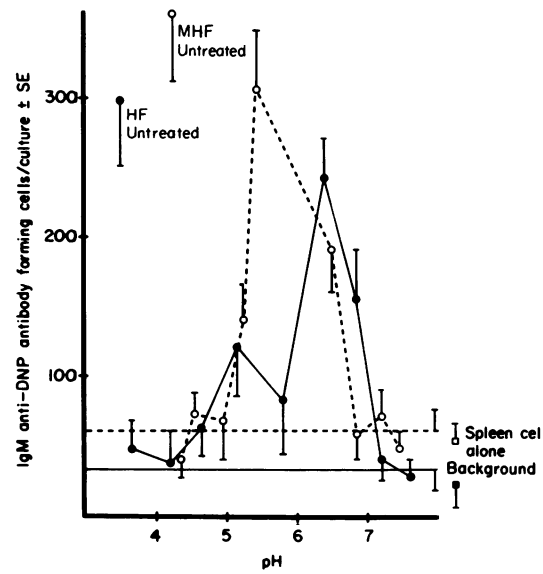

Figure 2. Isoelectric focusing of monkey (dotted line) and mouse (solid line) helper factor. Two hundred microlitres of culture supernatant from 5×10^6 T cells/ml were focused at 500 V for 17 h. After fractionation into 200 μl aliquots, functional activity was assayed at a dilution of $1:10^{-3}$. The presence of ampholines had no effect on helper activity.

The effect of temperature and enzymes on helper activity

The activity of both mouse and monkey helper factors was irreversibly destroyed after the fourth cycle of five freezing and thawing cycles (data not shown). As the helper factor titration curve shows activity at 10^{-1} and 10^{-2} dilutions (Lamb *et al.*, 1980), it seems likely that the activity of helper factor after four cycles of freezing and thawing was completely lost rather than diminished. The activity was also completely lost after a half hour incubation at 60° or 80°, but not at 37° (data not shown). The effect on helper activity of

Table 1. Effect of enzymes on the activity of mouse and monkey helper factors

Stimulus HF_{SA}	DNP-SA	Enzyme	Anti-DNP (AFC ± SE/culture)
B10.BR	+	Pronase	93 ± 18
B10.BR	+	Neuraminadase	107 ± 15
B10.BR	+	β-Glucuronidase	603 ± 17
B10.BR	+	Ribonuclease	580 ± 51
B10.BR	+	Tris chloride	613 ± 55
B10.BR	+	Tris acetate	647 ± 47
B10.BR	+	–	683 ± 52
Monkey	+	Pronase	73 ± 18
Monkey	+	Neuramindase	80 ± 25
Monkey	+	β-Glucuronidase	520 ± 56
Monkey	+	Ribonuclease	497 ± 46
Monkey	+	Tris chloride	543 ± 68
Monkey	+	Tris acetate	527 ± 68
Monkey	+	–	527 ± 66
–	+[a]	–	127 ± 20[a]
–	–[a]	–	87 ± 15[a]
–	DNP-PAA	–	2137 ± 434

0·25 ml samples of HF were incubated with agarose-insolubilized neuraminidase, β-glucuronidase, ribonuclease and CM-cellulose-insolubilized pronase for 0·5 h at 37°. Treated HF was then assayed in the cooperative culture at a final dilution of 10^{-3}.

The efficiency of B-cell stimulation was monitored with the T-independent antigen DNP-PAA.

Background (a) AFC were from spleen cells cultured without HF in the presence or absence of DNP-SA; helper factor alone without antigen gave similar values. *P* values are indicated as follows: ***$P < 0.001$, **$P < 0.01$; *$P < 0.05$.

incubation with degradative enzymes for 0·5 h is shown in Table 1. Ribonuclease and β-glucoronidase, enzymes degrading RNA and glucose conjugates, had no effect. The non-specific protease, pronase, and the sialic acid cleaving enzyme neuraminidase, both removed helper activity. These results suggest that glycoprotein moieties are essential for the activity of helper factors.

Serological analysis of factors

Monkey and mouse helper factors may be adsorbed to antisera to mouse helper factor but not antisera to suppressor factor (Fig. 3). These antisera recognize a 'constant' part of the antigen specific helper (or suppressor) molecule shared by antigen specific helper factors of mouse origin (Kontiainen & Feldmann, 1979). The result with monkey helper factor would imply that the sequences recognized by these antisera are highly conserved across a wide species barrier. Both factors bind to immobilized SA but not KLH, and also bind to antisera to SA (Fig. 4). These results demonstrate antigen specificity and the presence of antigenic determinants in the unfractionated factors.

To determine whether or not helper factor contained immunoglobulin determinants, mouse factor was reacted with rabbit antisera to mouse IgM and sheep antiserum to mouse Fab. Neither reagent adsorbed helper activity (data not shown). Rabbit antisera to the Fc portion of monkey IgM, IgG and IgA were used as adsorbents for monkey helper factor. Significant binding occured with anti-IgM sera. However, as the purity of the antiserum is not clear, further experiments need to be performed with other antisera against monkey IgM as well as monkey helper factors of different specificities to rule out contaminating antibodies.

Other laboratories have shown that antigenic determinants recognized by antisera directed against the products of the major histocompatibility complex are present in antigen specific helper (and suppressor) factors (Howie & Feldmann, 1977; Munro, Taussig,

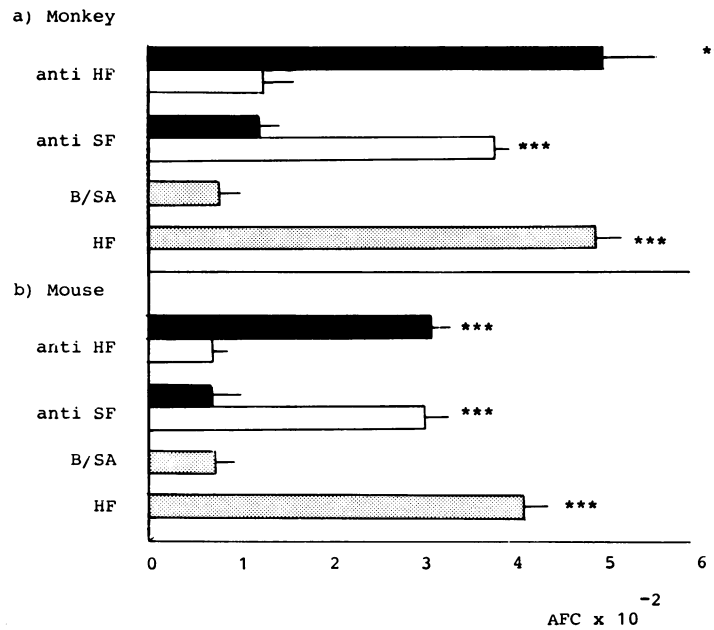

Figure 3. Immunoadsorbent analysis of (a) monkey and (b) mouse helper factor. Culture supernatants were diluted 1:10 and incubated with antigens or antisera coupled to sepharose. After removing unbound material (open histograms). Control experiments included unfractionated helper factor (HF) and B cells cultured in the presence (B/SA) or absence of antigen, both of which gave similar values. Statistics are indicated by standard errors (solid lines) and *P* values (asterisks, see legend to Table 1). *P* values are indicated as follows: ***$P < 0.001$, ** $P < 0.01$, *$P < 0.05$.

Campbell, Williams & Lawson, 1974; McDougal. Cort & Gordon 1977; Mudawwar, Yunis & Geha, 1978). Mouse helper factor was adsorbed out by anti-Ia^k but not by the control antiserum anti-Ia^s. Furthermore, anti I-A^k but not anti I-J^k absorbs activity (Fig. 6). Antisera to I-J^s also failed to absorb activity (data not shown). This suggests that the mouse factor carries determinants encoded by the I-A region of I.

DISCUSSION

Functional and structural analysis of rhesus monkey helper factor to an antigen implicated in caries may provide a model of *in vivo* cellular co-operation during immunization. To compare the primate factor with the widely studied murine equivalent, we have compared some of their properties by biochemical and serological methods. The approximate molecular weight of 70,000 for both factors is in broad agreement with the 25,000–80,000 range reported by other laboratories (Mudawwar *et al.*, 1978; Munro *et al.*,1974). The resolution of gel filtration is insufficient to establish whether the mouse and monkey factors are identical in size. They are distinguished by isoelectric focusing however, the monkey factor being more acidic. This technique may prove to be a useful tool in purifying factors for further analysis. An isoelectric point of 4·5 for a mouse helper factor had been reported by Shiozawa, Singh, Rubenstein & Diener (1977) and while this is not directly comparable to the factors described here, the acidic nature of these molecules may be a general phenomenon. The combined use of degradative enzymes and reagents such as pronase and periodate (McDougal & Gordon, 1977) of ficin and trypsin (Mudawwar *et al.*, 1978) with helper factors has shown that these probably contain glycoprotein moieties essential for activity. To examine the nature of these carbohydrate residues further, we have used the sialic acid cleaving enzyme neuraminidase to abolish the functional activity of monkey and mouse helper factors, the same results being achieved with heat and pronase treatment. These findings are consistent with the widespread occurrence of lymphocyte associated glycoproteins involved in cellular recognition (Snary, Barnstable, Bodmer & Crumpton, 1977).

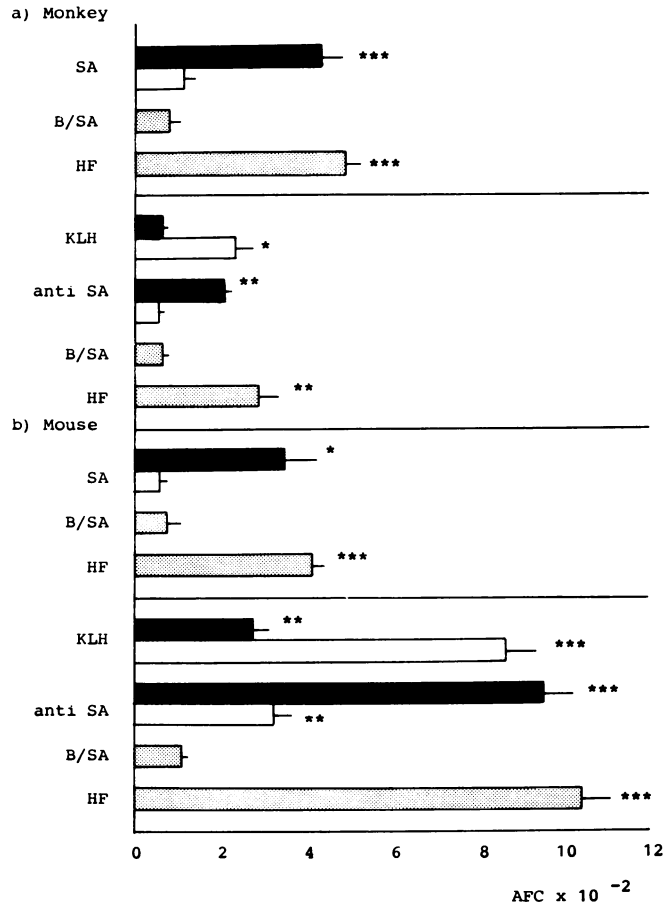

Figure 4. Immunoadsorbent analysis of (a) monkey and (b) mouse helper factor. For details, see legend to Fig. 3.

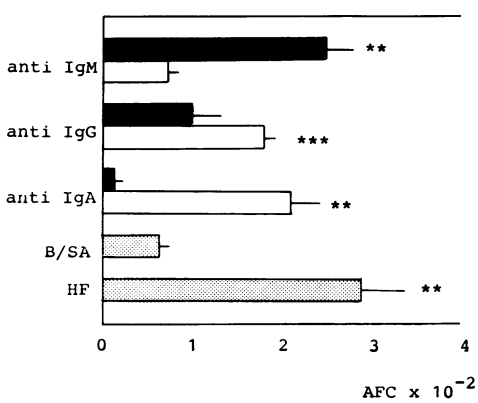

Figure 5. Immunoadsorbent analysis of monkey helper factor. For details see legend to Fig. 3.

From a practical viewpoint, the instability of both factors to freezing and thawing after four cycles is a useful guide for further studies. The availability of rabbit antisera reacting with mouse helper factors induced by different antigens has been used to argue for a constant region in factors as opposed to the variable region detected by mouse antisera to specific mouse factors (Kontiainen & Feldmann, 1979). The adsorption of primate helper factor to the rabbit antisera demonstrates a remarkable conservation of determinants over a wide species barrier and emphasises the similarity of the mouse and monkey system. The lack of reaction of helper factor with antiserum to a suppressor factor defines the specificity of the reaction.

In common with other antigen specific helper factors, an antigen-combining site and antigenic deter-

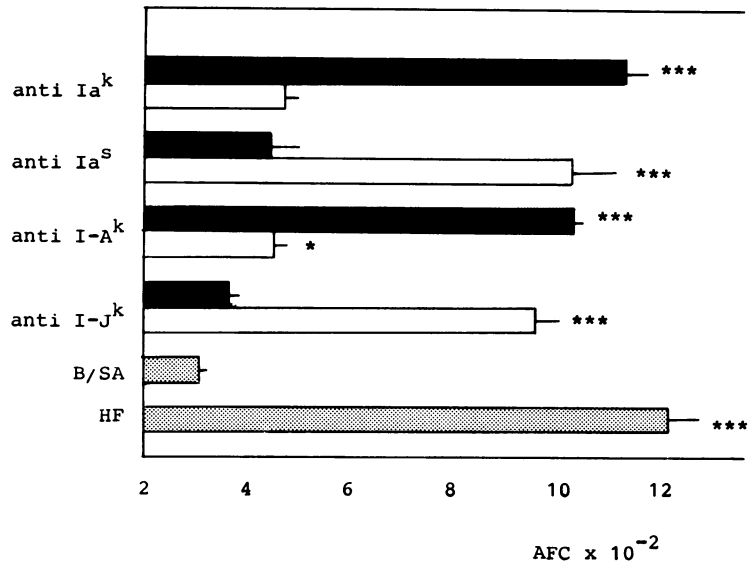

Figure 6. Immunoadsorbent analysis of mouse helper factor. For details see legend to Fig. 3.

minants were found in the factors described in this paper by adsorption of activity onto immobilized antigen and antiserum to that antigen (Taussig & Munro, 1974; Howie & Feldmann, 1977; McDougal & Gordon, 1977; Mudawwar *et al.*, 1978), It has been found that the binding of human helper factor to anti-SA serum is abrogated by further purification of the factor (Zanders *et al.*, in preparation). That the combining site could contain immunoglobulin determinants was suggested by Howie & Feldmann (1977) who could adsorb a mouse T-GAL specific helper factor to rabbit antisera to mouse IgM. Although other laboratories have failed to bind factors to antisera reactive against whole immunoglobulins or isolated chains (Taussig & Munro, 1974; McDougal & Gordon, 1977), including ourselves with antisera to polyvalent mouse immunoglobulin and Fab, the monkey factor is adsorbed to antisera to monkey IgM. As the epitopes recognized by this antiserum have not been identified, we are unable to state that the reactive element is immunoglobulin *per se*. It is quite possible that the antiserum reacts with carbohydrate determinants which cross react with lymphocyte surface antigens as shown for chicken antisera to mouse immunoglobulin (Ivanyi, Studwick & Makings, 1977) or rabbit antisera to human IgM (Merler, Gaten & de Wilde, 1974).

Although the results using antisera directed against the Fc portion of heavy chains are contradictory, it appears that the antigen combining site of specific factors might be encoded by a V_H pool. This is supported by the findings that antisera to the Fab portion of idiotypic antibodies absorb helper activity (Mudawwar *et al.*, 1978) or suppressor activity (Liew & Chan-Liew 1978) as do antisera to whole idiotypic antibodies (Germain, Ju, Kipps, Benacerraf & Dorf, 1979; Mozes & Haimovich, 1979).

Antigen-specific helper factors carry determinants recognized by antisera to the I region of the MHC, usually I-A (Munro *et al.*, 1974; Tada, Taniguchi & David, 1976; Howie & Feldmann 1977) or I-J (Howie, Parish, David, McKenzie, Maurer & Feldmann, 1979). Human factors to (TG)-A-L and KLH (A. Rees, personal communication), as well as tetanus toxoid Mudawwar *et al.*, 1978) carry HLA-D encoded DR determinants which are equivalent to murine Ia antigens. In this paper, the presence of I-A determinants has been demonstrated in mouse streptococcal antigen-specific helper factor; more recently, it has been possible to adsorb the monkey factor to a monoclonal antiserum to human Ia antigens (Lamb & Zanders, unpublished results). Such Ia determinants are probably not the same as the B-cell glycoproteins of 28,000 and 35,000 mol. wt (Cullen, Freed & Nathenson 1976), since the entire helper factor molecule has to accommodate an antigen-combining site as

well. The recent data of Howie *et al.* (1979) show that low molecular weight carbohydrate antigens may be the determinants recognized by commonly used antisera to mouse Ia. Further resolution of such structural problems awaits a more rigorous biochemical analysis of these lymphocyte factors.

ACKNOWLEDGMENTS

We wish to thank Ms C. Robsinson for excellent technical assistance, Dr M. Rittenberg for KLH, Dr M. Baltz for TNP-PAA, and Dr M. Feldmann for helpful discussions and the generous supply of anti-Ia antisera. This work was supported by a Medical Research Council grant, England, by U.S. Public Health Service grant No. 1.ROI.AI 13145-03 IMB (SK), and by the Sigrid Juselius Foundation in Finland (SK). JRL is in receipt of a Medical Research Council Training Fellowship.

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