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Cell-bound helper and suppressor factors in primate lymphocytes

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SUMMARY

Cell-bound helper and suppressor factors, on lymphocytes from Rhesus monkeys, were assayed by the indirect immunofluorescent method, using $F(ab')_2$ fragments of rabbit antisera to antigen specific secreted helper and suppressor factors. The rabbit antisera recognize the function related to the 'constant' region of secreted antigen specific helper or suppressor factor. Immunoadsorption studies suggest that the two antisera also recognize cell surface markers of helper or suppressor cell, for the activity of the anti-helper factor (HF) antiserum was adsorbed by cells from helper cell but not by suppressor cell induction cultures and the converse was found for the anti-suppressor factor (SF) antiserum. A significantly greater proportion of bound SF than HF was found in lymphocytes from controls and the converse or a greater proportion of HF than SF was found in lymphocytes from immunized monkeys. Furthermore, a significantly greater proportion of T-enriched cells bound anti-SF than anti-HF and the converse was found with T-depleted cells, suggesting that the assay detects the target cell as well as the cell of origin of these factors. A sequential *in vitro* comparison of cell-bound and secreted factors revealed that the highest number of cells which stained with anti-HF was on day 4 of the culture and this correlated with the highest secreted HF activity. However, cells which stained with anti-SF reached a peak on day 2, whereas maximal secreted SF activity was found on day 3. *In vivo* the kinetic relationships after immunization showed similar timings for the development of bound HF and SF, though the proportion of cells differed. However, the secreted HF after secondary immunization reached its highest level of activity on day 5, as compared with day 2 for cell bound HF. Both primary and secondary cellular but not antibody responses showed well defined bi-phasic responses and their significance as well as those of bound HF and SF in immunoregulation will need to be explored further.

INTRODUCTION

Regulation of the immune response is largely a function of interactions within the network of helper and suppressor cells or their soluble products (Benacerraf, 1980; Tada & Hayakawa, 1980). Helper and suppressor factors have been detected in mice (Tada, 1975; Kontiainen & Feldmann, 1976; Benacerraf, 1980; Tada & Hayakawa, 1980), Rhesus monkeys (Lamb, Kontiainen & Lehner, 1979, 1980) and man (Mudawwar, Yunis & Geha, 1978; Kantor & Feldmann, 1979; Kontiainen *et al.*, 1981a; Lehner *et al.*, 1981). On the basis of serological analyses a 'constant region' and 'variable

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region' have been postulated to form the structure of helper and suppressor factors (Kontiainen & Feldmann, 1979). Antibodies specific for the constant region of either helper or suppressor factor have been raised in rabbits. They adsorb specifically the HF activity or SF activity of mouse, monkey or human antigen specific factors (Kontiainen & Feldmann, 1979; Kontiainen *et al.*, 1981a, b; Lamb *et al.*, 1980; Zanders *et al.*, 1980).

Helper factor (HF) and suppressor factor (SF) are secreted by subsets of antigen-specific T lymphocytes; in mice helper cells bear antigen Ly-1^+ and suppressor cells Ly-2^+3^+ (Feldmann *et al.*, 1977; Cantor & Boyse, 1977). Secreted HF binds to adherent, macrophage-like cells (Howie & Feldmann, 1977) or B cells (Munro & Taussig, 1975). Secreted SF binds to activated T cells, notably T helper cells, preventing the function of T helper cells of the same antigenic specificity as that used to generate the SF (Kontiainen & Feldmann, 1978). Furthermore, suppressor extracts act to induce more SC (Tada & Hayakawa, 1980). Secreted HF or SF are not genetically restricted; human or monkey HF and SF act on and can be assayed by mouse cells (Kantor & Feldmann, 1979; Lamb *et al.*, 1979, 1980; Kontiainen *et al.*, 1981a; Lehner *et al.*, 1981). The objectives of this investigation were to induce helper and suppressor cells both *in vitro* and *in vivo*, to attempt to identify cell-bound helper and suppressor factors on lymphocytes by immunofluorescence and to relate the bound helper factor to secreted HF activity.

MATERIALS AND METHODS

Lymphocytes. Blood was collected from two groups of Rhesus monkeys; (1) nine control monkeys i.e. animals sham-immunized with saline and (2) 16 monkeys immunized with *Streptococcus mutans* cells or their protein antigens in Freund's incomplete adjuvant (FIA) or Adhydrogel (Russell & Lehner, 1978; Lehner *et al.*, 1981), 12–14 months after immunization. Peripheral blood lymphocytes were separated by Ficoll-Isopaque density gradient (Wilson & Kocvara, 1975). T-enriched cells were prepared from six monkeys by passing the cells over nylon wool adherence columns (Julius, Simpson & Herzenberg, 1973). These cells contained $6 \pm 0{\cdot}8\%$ of EAC rosetting cells. B-enriched cells were obtained by EAC rosetting with sheep erythrocytes (E) treated with rabbit antibody (A) to the erythrocytes, and mouse complement (C) (Urbaniak *et al.*, 1978). These cells showed $19{\cdot}8 \pm 4{\cdot}3\%$ of E-rosetting cells, as well as macrophage like cells, the proportion of which was not determined.

Preparation of antisera to helper and suppressor factors. Rabbit anti-SF and anti-HF were prepared to KLH specific mouse SF and HF as described previously (Kontiainen & Feldmann, 1979). Briefly, KLH specific HF or SF was first produced *in vitro* by culturing CBA spleen cells with an appropriate dose of KLH and purifying the factors from the cultures on KLH-immunoabsorbents. The eluted materials from the KLH columns were mixed with Freund's complete adjuvant (FCA) and injected into rabbits at weekly intervals for 6 months. The resulting antisera were specific to the functional subclass of the factors; they recognized either SF or HF and adsorbed out SF or HF activity. On the basis that rabbit antisera react with these factors, regardless of the antigen specificity or strain (or species) or origin of the factor, they define 'constant' region determinants of functional factor subclasses (Kontiainen & Feldmann, 1979; Kontiainen *et al.*, 1981b). The antisera were then adsorbed with normal human serum once or twice, until no precipitation was observed on immuno-electrophoresis with human serum. The sera were then digested with pepsin and the $F(ab')_2$ fragment separated on a sephadex G-150 column (Stanworth & Turner, 1978). $F(ab')_2$ fragments were used so as to avoid binding of the Fc piece of the undigested antibody to Fc receptors on the lymphocytes. Preliminary tests with the whole antibody showed considerable non-specific binding to lymphocytes.

Immunofluorescence assay for cell-bound helper and suppressor factors. One million lymphocytes were reacted with 50 μl of rabbit anti-helper or anti-suppressor factor serum at a concentration of 1 mg/ml for 30 min at 4°C and washed twice in TC199 medium, containing $0{\cdot}1\%$ sodium azide. The washed cells were then reacted with goat anti-rabbit FAb (IgG cut), conjugated with FITC (Northeast Biochemical Laboratories) for 30 min at 4°C, washed, mounted on microscope slides and viewed with a Leitz Orthoplan fluorescent microscope, using Ploem illumination. The number

of cells showing membrane fluorescence with the anti-helper or anti-suppressor antiserum were enumerated by counting about 500 cells. They were then expressed as the percentage of cells staining with either anti-HF or anti-SF, indicating cell-bound HF or SF. Any background staining of cells with the conjugate alone which varied between 0 and 1% was subtracted.

Serum antibody assay. IgG and IgM antibodies were assayed by immunofluorescence as described before (Lehner *et al.*, 1976). The titres are expressed as the highest dilutions of serum giving positive fluorescence of cells of *Streptococcus mutans*.

Preparation and assessment of helper and suppressor cells and their factors in vitro. To induce helper or suppressor cells monkey peripheral blood lymphocytes were cultured in modified Marbrook flasks for 4 days as described previously (Lamb *et al.*, 1979, 1980). Ten nanograms of the streptococcal antigen (SA) per ml was added to 5×10^6 viable cells per ml to induce helper cells and 100 μg of SAI/II per ml was added to 5×10^6 cells per ml to induce suppressor cells. Cells staining with anti-HF and anti-SF were enumerated on days 0, 1, 2, 3 and 4 of the culture in both the low and high concentrations of SA stimulated cell cultures. A parallel assessment of the optimum time required to induce helper and suppressor functions was carried out over 3, 4 and 5 days. The putative helper or suppressor cells were then stimulated with a further dose of 0·1 μg of SA per ml for 1 day and the supernatants were assayed for HF or SF. HF was assayed *in vitro* by adding it to 10^7 normal mouse spleen cells stimulated with 0·1 mg/ml of DNP-SA. The SF was assayed in cooperative cultures of 3×10^5 *in vitro* induced SA specific mouse helper cells and normal mouse spleen cells cultures with 0·1 μg/ml of DNP-SA for 4 days. The number of IgM antibody forming cells (AFC) was assessed on day 4 of the culture by the modified Cunningham assay (Lamb *et al.*, 1979). All the cooperative cultures were performed in triplicates.

The development of cell-bound helper and suppressor factors and secreted helper function after in vivo *immunization.* Three control monkeys (about 4 kg in weight) were immunized subcutaneously with 100 μg of SA in FIA and they were then re-immunized 48 days later, with the same amount of antigen and adjuvant. Serial examinations were performed on peripheral blood lymphocytes to detect cell-bound HF and SF and for their ability to produce helper activity. The helper factor activity was assayed by culturing 5×10^6 peripheral blood lymphocytes with 1 to 1000 ng of SAI/II for 1 day only (Lehner *et al.*, 1981) and the supernatants were tested for released HF activity as described previously (Lamb *et al.*, 1979). The results were expressed as the net AFC in which the AFC without HF were subtracted from the AFC with HF. Serum IgG and IgM antibodies were determined to *S. mutans* as described above.

Immunoadsorption and inhibition studies for cell-bound and SF specificity. Inhibition studies were carried out with antisera to rabbit anti-human IgG, IgM (Behringwerke) or fetal calf serum and monoclonal antisera to the non-polymorphic regions of HLA-DR (DA2), kindly supplied by Dr M. Crumpton, and to β_2M by Dr A. Sanderson. Aliquots of 50 μl of the anti-Ia (DR) or anti-β_2M antisera (2 mg/ml) and anti-IgG or anti-IgM antisera (4 mg/ml), were incubated with equal volumes of the F(ab′)$_2$ fragments of anti-HF or anti-SF antisera for 30 min at 4°C and then tested by immunofluorescence.

The F(ab′)$_2$ fragments of the antisera to HF and SF were each adsorbed separately with cells from cultures designed to produce helper or suppressor cells. These cells were prepared as described above by culturing lymphocytes with a low or high dose of SAI/II for 4 days. Untreated lymphocytes were also used for adsorption as a further control. Samples of 0·5 ml of the antisera were mixed with 10×10^6 helper, suppressor or untreated cells and rotated on a blood mixer for 60 min at room temperature. The cells were centrifuged at 100 r.p.m. and the unbound material was then tested on freshly separated lymphocytes.

Statistical analysis. Students' *t*-test for two means or as appropriate the paired t test was used. Spearman's rank correlation was applied to test if the ranking between the HF and SF was significant.

RESULTS

Cell-bound helper and suppressor factors and secreted HF in lymphocytes from control and immunized monkeys

Cell-bound HF was found in 0·6–3·6% and SF in 1·3–4·1% of lymphocytes from control monkeys

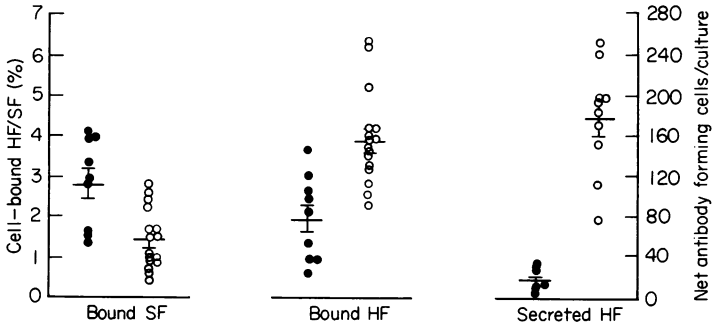

Fig. 1. Mean (±s.e.) cell-bound helper factor (HF) and suppressor factor (SF) in controls (●) and immunized (○) monkeys.

(Fig. 1). A significantly greater number of cells stained with anti-HF in the immunized (3·9±0·3%; mean±s.e.) than in the control (1·9±0·35%) group ($t=4·278$, $P<0·0001$, d.f. 23). However, a smaller number of cells stained with anti-SF in the immunized (1·4±0·2%) than control (2·8±0·4%) groups ($t=3·726$, $P<0·005$, d.f. 23). Furthermore, a significantly greater number of cells stained with anti-SF (2·8±0·4%) than anti-HF (1·9±0·35%) in the controls ($t=4·537$; $P<0·005$; d.f. 8). A similar analysis of bound HF and SF in lymphocytes from immunized monkeys revealed the converse; the number of lymphocytes which stained with anti-HF (3·9±0·3%) was significantly greater than those with anti-SF (1·4±0·2%; $t=12·362$; $P<0·0001$; d.f. 15). Furthermore, a significant correlation was found between the percentage of cells stained with anti-HF and with anti-SF, both in controls ($r=0·9146$, $P<0·001$, d.f. 7) and in the immunized monkeys ($r=0·6831$, $P<0·01$, d.f. 14). The secreted HF, expressed as the number of antibody forming cells per culture (Fig. 1) was clearly much higher in the immunized ($177·4±16·9\times10^7$ cells) monkeys, compared with the control group ($18·0±4·2\times10^7$ cells).

The distribution of HF and SF on T cell and B cell enriched population

Cell-bound HF (Fig. 2) was found in a significantly greater number of B-enriched than T-enriched populations of cells from immunized monkeys ($t=5·7981$; $P<0·005$; d.f. 5). In contrast cell-bound SF was significantly more frequent in T-enriched than T-depleted populations ($t=4·8704$; $P<0·005$; d.f. 5). The greatly increased ratio of the percentage of HF:SF in B-enriched cells and SF:HF in T-enriched cells can also be clearly seen in Fig 2; the two ratios are given for the same population of cells.

Specificity of cell-bound HF and SF

The specificity of the anti-helper and anti-suppressor factors antisera, can be seen on adsorption of the anti-HF with cells in the helper cell induction culture but not by those of the suppressor cell induction culture (Fig. 3). Similarly anti-SF was adsorbed by cells from the suppressor cell induction culture but not by those from the helper cell induction culture. However, untreated cells failed to reduce the percentage of lymphocytes reacting with anti-HF or anti-SF antiserum.

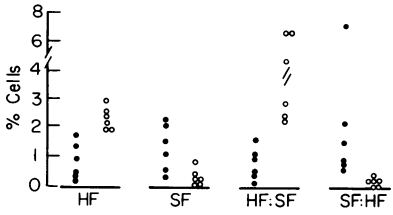

Fig. 2. Bound helper factor (HF) and suppressor factor (SF) on T-enriched (T = ●) and B-enriched (B = ○) cell populations.

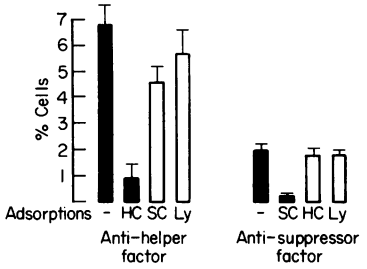

Fig. 3. Adsorption of anti-helper factor or anti-suppressor factor F(ab')$_2$ antisera with putative helper (HC), suppressor cells (SC) or lymphocytes (Ly); the means (±s.e.) are given for five different experiments.

Inhibition of immunofluorescence of the anti-HF antiserum by anti-IgG, -IgM or -FCS showed only minor changes (Table 1). However, greater inhibition of staining of the anti-HF but not anti-SF antisera were found with the anti-Ia and anti-β_2M ($t=4{\cdot}335$, $P<0{\cdot}05$, d.f. 2) ($t=3{\cdot}308$, $P<0{\cdot}1$, d.f. 2) antisera (Table 1).

Kinetics of development of cell-bound and secreted HF and SF in vitro

Stimulation of lymphocytes with a low or high dose of SA showed a decrease in bound HF or SF on day 1, detectable by both the anti-HF and anti-SF antisera (Fig. 4). However, lymphocytes stimulated with a low dose of SA, showed a daily increase in the number of lymphocytes staining with anti-HF but not anti-SF, up to the culture duration of 4 days. In contrast a high dose of SA induced an increase in lymphocytes staining with anti-SF and the highest numbers were reached by 2 days, without a change in lymphocytes staining with anti-HF (Fig 4). To demonstrate the optimal time for helper cell induction assayed by the ability to secrete HF, monkey helper cells (induced by the low dose of SA) from 3, 4 and 5 day cultures were restimulated with 0·1 μg of SA for 1 day. The cell-free supernatant (helper factor) was added to mouse spleen cells and anti-DNP AFC were assayed on day 4. The optimal induction time for helper cells was 4 days (Fig 4) and this was comparable with the results from the cell-bound HF experiment. Similar experiments were carried out to determine the optimal time for suppressor cell induction, using a high dose of SA and incubating the cells for 2, 3 and 4 days. The cells were then restimulated with 0·1 μg SA for one day and the putative suppressor factor was added to mouse spleen cells in the presence of mouse HC to SA. Anti-DNP AFC were assayed on day 4. Optimal suppression was observed on day 3 and this compares with day 2 observed for cell-bound SF.

Table 1. Inhibition of anti-helper factor (HF) or anti-suppressor factor (SF) with antisera

	Mean (±s.e.) per cent cell-bound HF or SF			
	Inhibition with antisera to			
Antisera	Untreated	IgG	IgM	FCS*
Helper Factor	5·4 (0·7)	3·2 (0·5)	4·7 (0·5)	4·9 (2·1)
Suppressor Factor	2·7 (0·7)	2·6 (0·15)	2·3 (0·1)	3·4 (1·1)
	Untreated	Ia	β_2M	
Helper Factor	2·7 (0·9)	1·1 (0·6)†	1·1 (0·4)‡	
Suppressor Factor	1·6 (0·1)	0·9 (0·7)	1·5 (0·3)	

*Fetal calf serum
†t4·335, d.f. 2, $P<0{\cdot}05$
‡$t=3{\cdot}308$, d.f. 2, $P<0{\cdot}1$

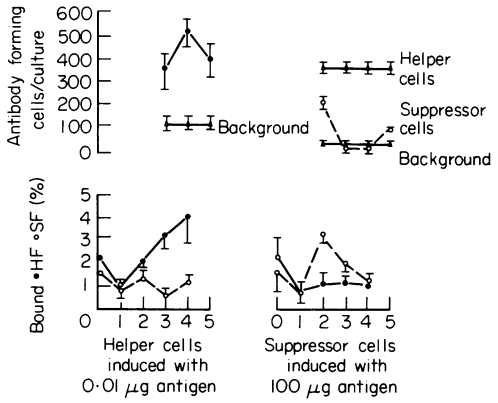

Fig. 4. Sequential comparison of bound and secreted helper and suppressor factors induced *in vitro*; the means (±s.e.) are given for three different experiments.

Development of cell-bound HF and SF in primary and secondary immunization of monkeys

Primary immunization with SA resulted within 2 days in a brisk increase in the number of cells stained with anti-HF and anti-SF (Fig. 5). Thereafter a rapid decrease occurred in the number of cells staining with anti-HF but to a relatively lesser extent in those staining with anti-SF, so that by day 5 there was the same or slightly increased number of cells with bound SF than HF. This was reversed by day 8, when the number of cells with bound HF or SF were comparable to those found before immunization. The changes in secondary immunization were similar to those found in primary immunization, except that cells with bound HF almost reached their peak by day 1, unlike cells with bound SF which reached their peak only by day 2. As blood was not examined on day 1 during primary immunization, it is not clear whether the latter relationship is specific to secondary immunization. A decrease in cells with both bound HF and SF followed and since the rate appeared to be slower for the latter, this resulted in a relatively higher number of bound SF than HF at 5 days. In both primary and secondary immunization a second peak of cells with bound HF and SF appeared about day 14 and declined within about 2 days, in one monkey, but lasted slightly longer in the other.

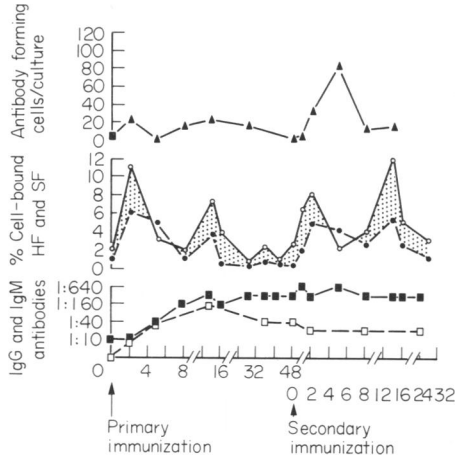

Fig. 5. Sequential relationship between cell-bound helper (HF) and suppressor factors (SF), secreted HF and serum antibodies after immunization of one monkey with 100 μg of streptococcal antigen. Two other monkeys showed comparable results. (▲——▲ = secreted HF; ○——○ = bound HF; ●——● = bound SF; ■——■ = IgG; □——□ = IgM).

The assay for secreted HF showed that 2 days after primary immunization lymphocytes showed a slight increase of 20 antibody forming cells (AFC) per 10^7 lymphocytes, as compared with those before immunization. However, in secondary immunization, although a similar increase in secreted HF was detected on day 2, there was then a significant increase by day 5 of 83 AFC/10^7 cells and this lasted less than 3 days.

In primary immunization, IgG antibodies also begin to increase from about day 2, with a rapid rise in titre by day 5, reaching a maximum of about 1:320 by day 14. IgM antibodies appeared at about the same rate as IgG antibodies but reached a lower titre of about 1:160 by day 14. In secondary immunization, there was no significant increase in the IgG antibody titre from a level of 1:320 to 1:640. However, IgM antibodies fell to a titre of 1:20 by week 28.

DISCUSSION

Cell-bound helper and suppressor factors were detected in up to 3·6% of peripheral blood lymphocytes of unimmunized and up to 6·7% of those immunized Rhesus monkeys tested about 1 year after immunization. There was a significantly greater number of cells with bound SF than HF in controls, but the proportion of cells with bound HF increased significantly over those with bound SF in immunized monkeys (Fig. 1). It is noteworthy that a significant correlation was found between cell-bound HF and SF in both the control and immunized monkey populations.

Immunofluorescence studies of T and B cell enriched populations showed that anti-SF stained a significantly greater proportion of the T-enriched and anti-HF of the B-enriched populations (Fig 3). Both HF and SF are produced by T cells in mice (Feldmann & Basten, 1972; Kontiainen & Feldmann, 1976), in monkeys (Lamb *et al.*, 1979, 1980) and in man (Kantor & Feldmann, 1979; Kontiainen *et al.*, 1981a). It is possible that the immunofluorescence assay for cell-bound HF and SF identifies both the target cell and the cell of origin of HF and SF. The target for secreted SF appears to be the T helper cell (Kontiainen & Feldmann, 1978; Lamb *et al.*, 1979) and for HF either the macrophage or B cell (Howie *et al.*, 1979; Lamb *et al.*, 1980). The relationship between T cell production of HF or SF, their expression on the T helper or suppressor cell membrane, the secretion or shedding of these factors and the target receptors will need careful examination.

The specificities of rabbit antisera to HF and SF for the corresponding constant regions of HF and SF have been established previously by immunoadsorption, rosetting and T cell plaque assay with mouse helper and suppressor cells and factors (Kontiainen & Feldmann, 1979; Kontiainen *et al.*, 1981b; Feldmann *et al.*, 1981) and monkey helper and suppressor factors (Zanders *et al.*, 1980; Lamb *et al.*, 1979). This has also been found with human HF (Lehner *et al.*, 1981) and has now been extended to the cell-bound HF and SF (Fig. 3). These xeno-antisera recognize the constant regions of HF or SF, both in the secreted and cell-bound state. They also recognize specifically the cell surface markers of helper and suppressor cells, for the anti-HF antiserum was adsorbed by helper cell but not by suppressor cell induction culture or unstimulated lymphocytes and the converse was found for the anti-SF antiserum (Fig. 3). However, no significant inhibitions were observed with the antisera to IgG, IgM, fetal calf serum, or β_2M.

Sequential *in vitro* examination for cell-bound and secreted HF revealed that the maximal number of cells with bound and secreted HF activity was on day 4 (Fig 4). A similar study of SF showed that both bound and secreted SF reached an optimal value earlier, and this was on day 2 for the bound SF and on day 3 for secreted SF (Fig. 4). The interpretation of the difference of 1 day between optimal formation of bound and secreted SF is not clear and suggests that suppressor cells produce the factor within the first 2 days but there is some delay in secretion or shedding the SF from the cell membrane.

In vivo development of bound HF and SF, secreted HF and serum antibodies was followed after primary and secondary immunization of monkeys with 100 μg of SA in FIA (Fig. 5). A brisk increase in both cell-bound HF and SF occurred within 2 days of immunization, followed by a fall in both cell-bound factors by 5 days, reaching baseline by 8 days. This pattern was similar for primary and secondary immunization, except that it may have been faster in the latter. These findings

suggest that immunization elicits both helper and suppressor cell activity and that no sooner are the cellular immune responses induced than they are suppressed, so as to limit their duration.

A second peak of activity of cell-bound HF and SF was observed on day 14 to 16 after both primary and secondary immunization. The interpretation of this must await further analysis but there are at least 3 possibilities; (a) feedback suppression of antibody synthesis by 19S antibodies has been proposed in mice to be responsible for the cyclical 19S antibody responses (Britton & Möller, 1968, Britton, Wepsic & Möller, 1968); (b) release from cellular suppression may take from day 5 to about day 14 to become evident functionally, with a new burst of cellular activity; (c) alternatively, recruitment of new cells, helper and suppressor may take about 10 days to take effect.

It is not clear what effect, if any, the rise in IgG and IgM antibody titres might have on the cellular responses. As both classes of antibodies are significantly raised by 5 days, they could play a part in suppressing the cellular responses. However, this is unlikely to account for the cyclic nature of the cell-bound HF and SF, in the absence of similar fluctuations in the antibody titres. Indeed, from day 14 of primary immunization and during the entire period of secondary immunization the IgG antibody titre remained at a level between 1:320 and 1:640. It should be noted that IgM antibodies fell, predictably, after day 14 and the fall in titre continued in spite of secondary immunization (Fig. 5).

Secreted HF from lymphocytes of immunized monkeys induce a significantly greater number of antibody forming cells than those from controls (Fig. 1). This was similar to the findings with cell-bound HF. The results suggest that immunization increases helper activity, whether assayed by the cell-bound HF which is not antigen specific (Kontiainen & Feldmann, 1979) or by secreted HF which is strictly antigen specific (Lamb *et al.*, 1981). The sequential pattern of development of secreted HF is of interest as there was only a slight increase during primary immunization, but this was rather marked on day 5 of secondary immunization. Thus, the ability to release HF from *in vivo* induced putative HC follows the development of cell-bound HF by about 3 days and this makes reasonable biological sense.

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