MOLECULAR AND CELLULAR BASIS OF HEPATIC FIBROGENESIS IN EXPERIMENTAL SCHISTOSOMIASIS MANSONI INFECTION

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Morbidity in schistosomiasis mansoni occurs primarily as a result of the complications of hepatic fibrosis. Yet, the pathogenesis of schistosomal hepatic fibrosis is poorly understood. The fact that the hepatic egg granuloma is the hallmark of this infection suggests a potential role for granulomatous inflammation in hepatic fibrogenesis. Our studies in a murine schistosomiasis model indicate that hepatic granuloma cells secrete a variety of fibrogenic cytokines that may initiate the scarring process. Among these cytokines, we identified a novel protein that we designated fibroblast stimulating factor-1 (FsF-1). FsF-1 is a lymphokine that can stimulate fibroblast growth and matrix synthesis. A notable feature of hepatic fibrosis in this model is that production of FsF-1 and other granuloma-derived fibrogenic cytokines is down-regulated in chronic infection, an event that may be under immunological control. The spontaneous reduction of FsF-1 secretion presumably accounts for reduced scar formation late in infection of mice. In the context of relevant clinical studies, our findings engender the hypothesis that Symmer's fibrosis may develop in a small subpopulation of individuals as a result of immunogenetically-determined dysregulation of fibrogenic cytokine production.

Key words: schistosomiasis – hepatic fibrogenesis

The description by Symmer (1904) of "clay pipe-stem" hepatic fibrosis in schistosomiasis mansoni lead the way to our current understanding of the clinical basis of serious morbidity in this disease. A striking feature of the epidemiology of schistosomiasis is that only a small subpopulation (< 10%) of individuals infected with *Schistosoma mansoni* develop Symmer's fibrosis; most remain relatively disease-free (reviewed by Chen & Mott, 1988). This fact suggests that once it is possible to identify the individuals at risk for developing severe liver fibrosis, specifically targeting them for intervention might ultimately prove to be an effective disease-control strategy.

Extensive efforts have been made to determine risk factors associated with liver fibrosis in schistosomiasis mansoni (see Chen & Mott, 1988). Neither parasite virulence factors nor genetically-determined host factors have been uncovered that are consistently associated with an increased relative risk of developing pipe-stem fibrosis. Until recently, efforts to identify risk factors were stymied in part by imprecise non-invasive means of objectively assessing liver fibrosis. However, the application of hepatic ultrasonography (see, for example, Abdel-Wahab et al., 1989) has substantially strenthened more recent research efforts and will prove indispensible in the future.

The biological basis for liver fibrosis in schistosomiasis can now also be analysed, in light of new insights into fibrogenesis generally (Postlethwaite, 1983; Evered & Whelan, 1985; Freundlich et al., 1986). Originally considered a pathological "dead end" and therefore neglected, fibrosis has more recently enjoyed active investigation. From the research has emerged a clear perspective that fibrogenesis is a highly regulated, dynamic, and potentially reversible process. The influence of a variety of cytokines on fibroblast functions (Freundlich et al., 1986; Agelli & Wahl, 1986) has drawn particular attention to the role of these soluble factors in pathological fibrogenesis.

This paper reviews our studies which indicate that the egg granuloma is the source of

fibrogenic cytokines; that these cytokines are produced as a result of host responses to egg antigens; and that the cytokines are actually responsible for inducing fibrosis in vivo. In concluding, a hypothesis will be proposed suggesting a biological basis for the selection of schistosomiasis patients who develop liver fibrosis.

MATERIALS AND METHODS

Granuloma isolation and culture – To assess whether hepatic fibrosis might be influenced by cytokines produced by granuloma cells, hepatic egg granulomas were isolated and incubated in serum-free medium, and cell-free culture supernatants were assayed for relevant biological activities. C57BL/6 mice were infected by intraperitoneal injection with about 50 cercariae of *S. mansoni* (Puerto Rico strain). Routinely at 8 weeks of infection (or at other times as specifically indicated), mice were euthanized and liver tissue was aseptically retrieved and subjected to mild homogenization in a Waring Blendor. Intact granulomas were isolated from the homogenate by serial sedimentation (Pellegrino & Brener, 1956) and were incubated at 37 C for 24 h in serum-free medium RPMI supplemented with L-glutamine and antibiotics (Wyler et al., 1978). Culture supernatants ("granuloma supernatant") were then prepared by centrifugation and filtration to remove cells and particulate debris. The histopathological features, parameters of viability, and kinetics of cytokine production of these granulomas has been reported (Wyler et al., 1981). In selected experiments, granulomas were dissociated by collagenase treatment, and macrophages or lymphocytes were isolated from the dispersed cell population (by adherence to plastic or by fluorescence-activated cell sorting, respectively).

Fibroblast cultures – Dermal fibroblast cultures were prepared from explants of human foreskin, and fibroblasts retrieved by trypsinization of low-passage cultures were assessed for selected in vitro responses to granuloma supernatants (Wyler et al., 1978, 1981). Comparative studies revealed that the responses of human cells was qualitatively similar to those of rodent fibroblasts, and yet were easier to maintain.

Biological assays

Fibroblast chemotaxis was assayed (in collaboration with Dr Arnold E. Postlethwaite) in modified Boyden chambers by published methods (Postlethwaite et al., 1978).

Fibroblast proliferation was measured by assessing the magnitude of ^{3}H thymidine incorporation in fibroblasts incubated for 24 h with granuloma supernatant (or other additives) and then pulsed for 4 h with the radionucleotide. Details of the methods have been published (Wyler et al. 1978, 1981).

Collagen synthesis was quantified in chick calvaria cultures (Wyler et al., 1987) or fibroblasts (Prakash et al., 1991) by assessing the incorporation of ^{3}H proline into collagenase-sensitive protein in the presence and absence of granuloma supernatant or its fractions.

Fibronectin production by fibroblasts was measured in an ELISA by published methods (Wyler et al., 1987). Immunoreactive fibronectin was quantified in granuloma supernatant and in supernatants of fibroblasts stimulated with granuloma products. The *production of hyaluronan* (another important matrix constituent) was determined by measuring the incorporation of ^{3}H-acetate into hyaluronidase-sensitive material (Prakash et al., 1991).

Fractionation of granuloma supernatants was carried out in an effort to characterize and then purify granuloma-derived fibrogenic factors. Gel filtration and affinity chromotography, preparative isoelectric focusing, and polyacrylamide gel electrophoresis were employed by standard methods (Wyler & Rosenwasser, 1982; Prakash & Wyler, 1991).

RESULTS

Egg granulomas produce fibrogenic cytokines – The fundamental biological processes that underlie fibrogenesis include: the local recruitment of fibroblasts (to the scar site), fibroblast hyperplasia (proliferation), excess extracellular matrix production by fibroblasts, and remodelling of extracellular matrix (with degradative enzymes) (Fig. 1). Granuloma culture supernatants contain biological activity that can influence all these processes.

Crude (unfractionated) granuloma supernatant contains a chemoattractant for fibroblasts (Wyler & Postlethwaite, 1983). When placed on one side of a permeable membrane, the supernatant induces net migration of fibroblasts along a chemical gradient (Fig. 2). The
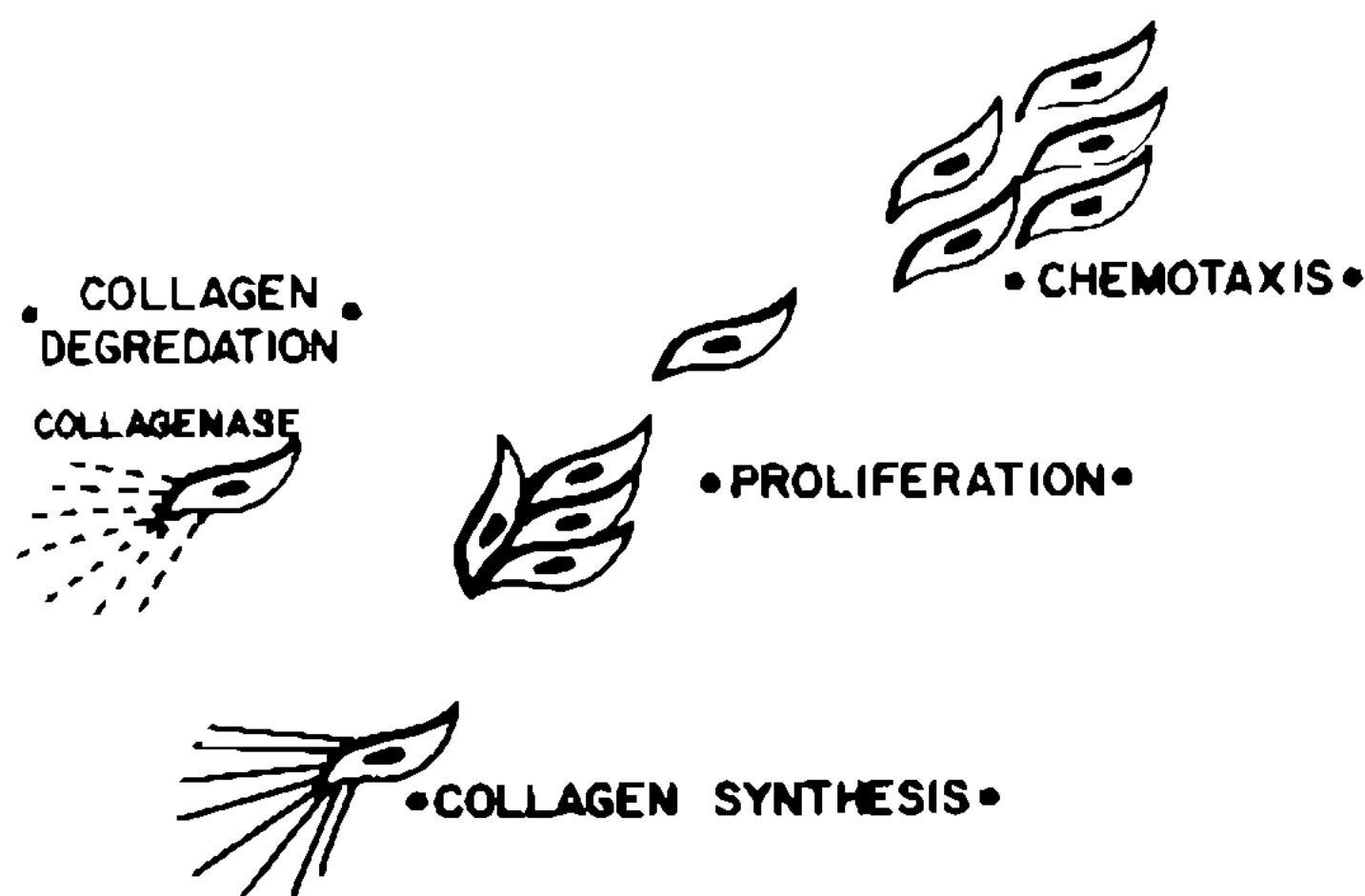
Fig. 1: the elements of fibrogenesis. In response to locally-produced fibrogenic peptides, fibroblasts are recruited by chemotaxis, stimulated to proliferate, and induced to produce excess extracellular matrix of which collagen is a major component. Since collagen degredation is also part of the process, the balance between production and degredation determines the net deposition.

chemoattractant is large (Mr≈400 kDa) and has the same isoelectric point (pI) as plasma fibronectin. When antifibronectin antibody is added to fibronectin (a known fibroblast chemoattractant [Postlethwaite et al., 1981; Tsukamoto et al., 1981]) or granuloma supernatant, but not to collagen (also a known chemoattractant [Postlethwaite et al., 1981]), it specifically abrogates the chemotactic response. Since granuloma culture supernatants contain fibronectin (Wyler, 1987), the cumulative results point to fibronectin (or a large fragment thereof) as the molecular basis of the chemotactic activity in the granuloma supernatants. One cell source of the chemoattractant within the granuloma is the macrophage population. When isolated from egg granulomas, these cells have phenotypic and functional features of "activated" cells (Wyler et al., 1984) and spontaneously secrete a fibroblast chemoattractant that has the same features (apparent identity to fibronectin) as the chemoattractant isolated from culture supernatants of whole, intact granulomas (Wyler & Postlethwaite, 1983).

Crude granuloma culture supernatants also stimulate fibroblast growth (Wyler et al., 1978, 1981). The granuloma-derived growth factor is distinct from the chemoattractant, however. It can be purified to homogeneity from granuloma supernatants by a simple two-step procedure involving gel filtration followed by heparin-affinity chromatography (Prakash & Wyler, 1991). The biologically-active fractions separated by gel filtration (Biogel P30) are pooled
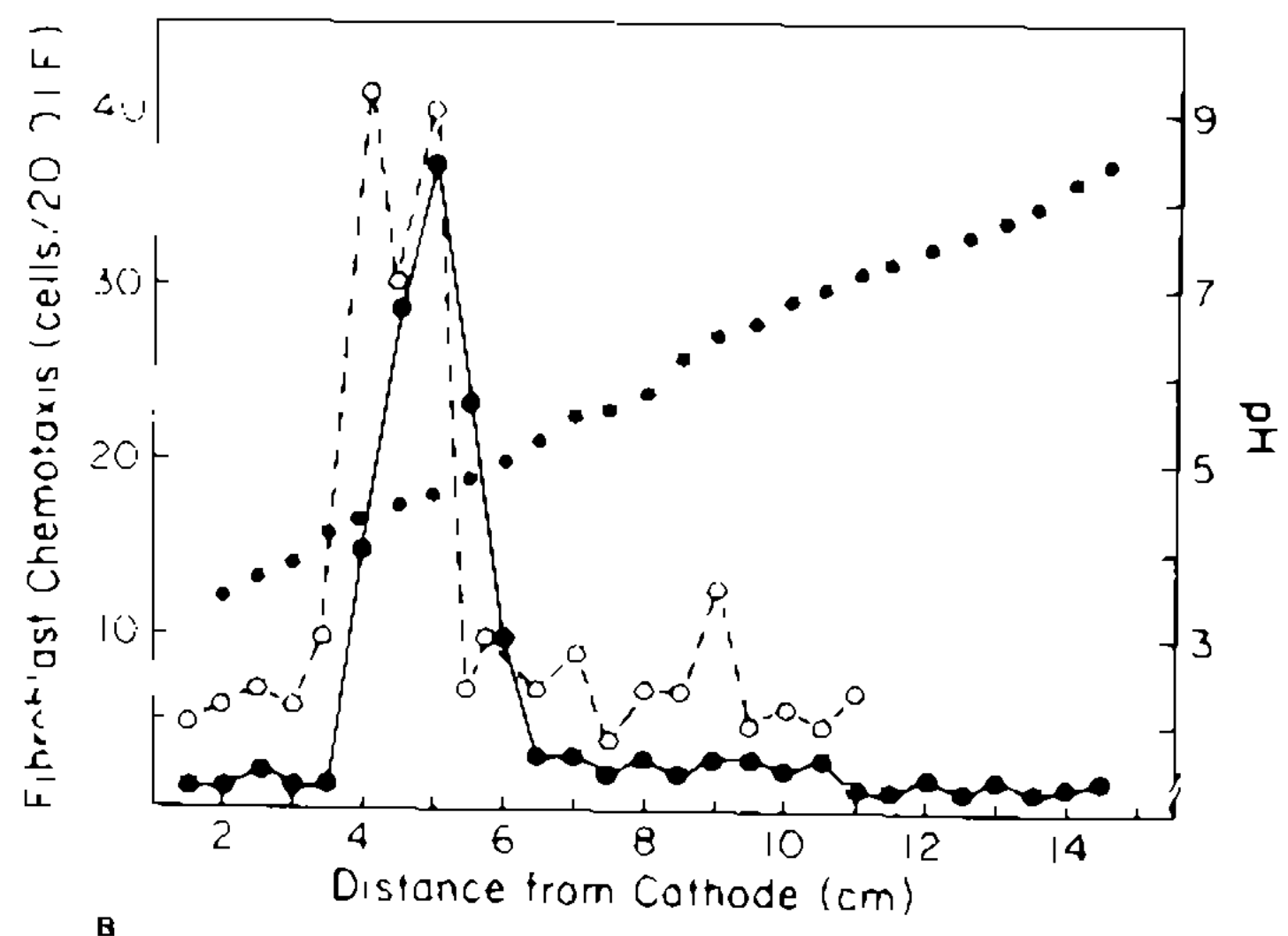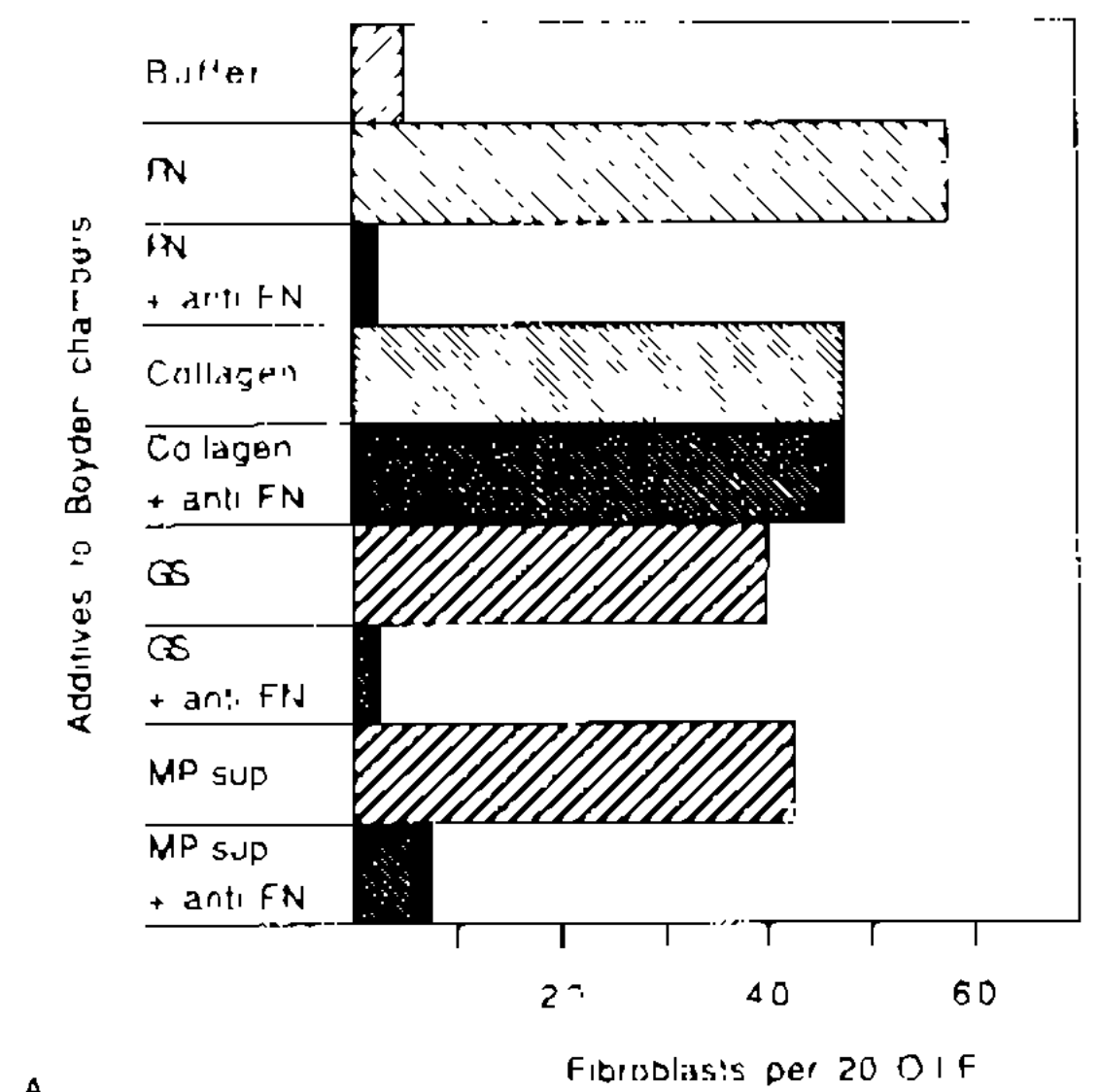

Fig. 2: stimulation of fibroblast chemotaxis by products of schistosomal egg granulomas. Fibroblasts were placed in the upper compartment of modified Boyden chambers and the indicated additives were placed in the lower compartment. After incubation, the numbers of fibroblasts that migrated through polycarbonate filters toward the lower compartment was quantified microscopically and expressed per 20 oil immersion fields (O.I.F.). Mean of triplicate determinations is shown: SEM < 10%. A: Chemotactic response to: FN, fibronectin; anti FN, rabbit antifibronectin IgG; GS, granuloma supernatant; MP sup, cell-free supernatant from cultures of isolated granuloma macrophages. B: Chemotactic response to fractions of granulomas supernatant (closed symbol) or plasma fibronectin (open symbol) subjected to preparative isoelectric focussing. Note that biological activity was present in fractions of granuloma supernatant and of fibronectin that had pI ≈ 4-4.8. (Reproduced from Wyler, D. J., & Postlethwaite, A. E., 1983, J. Immunol., 130: 1371-1375, with permission of the journal).

and incubated with heparin-Sepharose (0.15M NaCl; pH 7.2). The non-adsorbed material ("fall-through") is removed by washing, and the heparin-binding fraction is eluted with a gradient of NaCl (0.5-2M) or with 1.5M NaCl in a batch preparation. The fibroblast mitogenic activity is detected only in the fraction

that elutes with 1.5 M NaCl (Fig. 3). This two-step purification results in about a 10,000-fold increase in specific activity (Prakash & Wyler, 1991). When subjected to SDS-PAGE under reducing conditions, this fraction contains only one silver nitrate-stainable band, which migrates with an apparent Mr ≈60 kDa. This single band contrasts with the very large number of bands identified in crude granuloma supernatant (Fig. 4). As noted (below), the granuloma-derived fibroblast mitogen purified in this manner is an apparently novel growth factor.
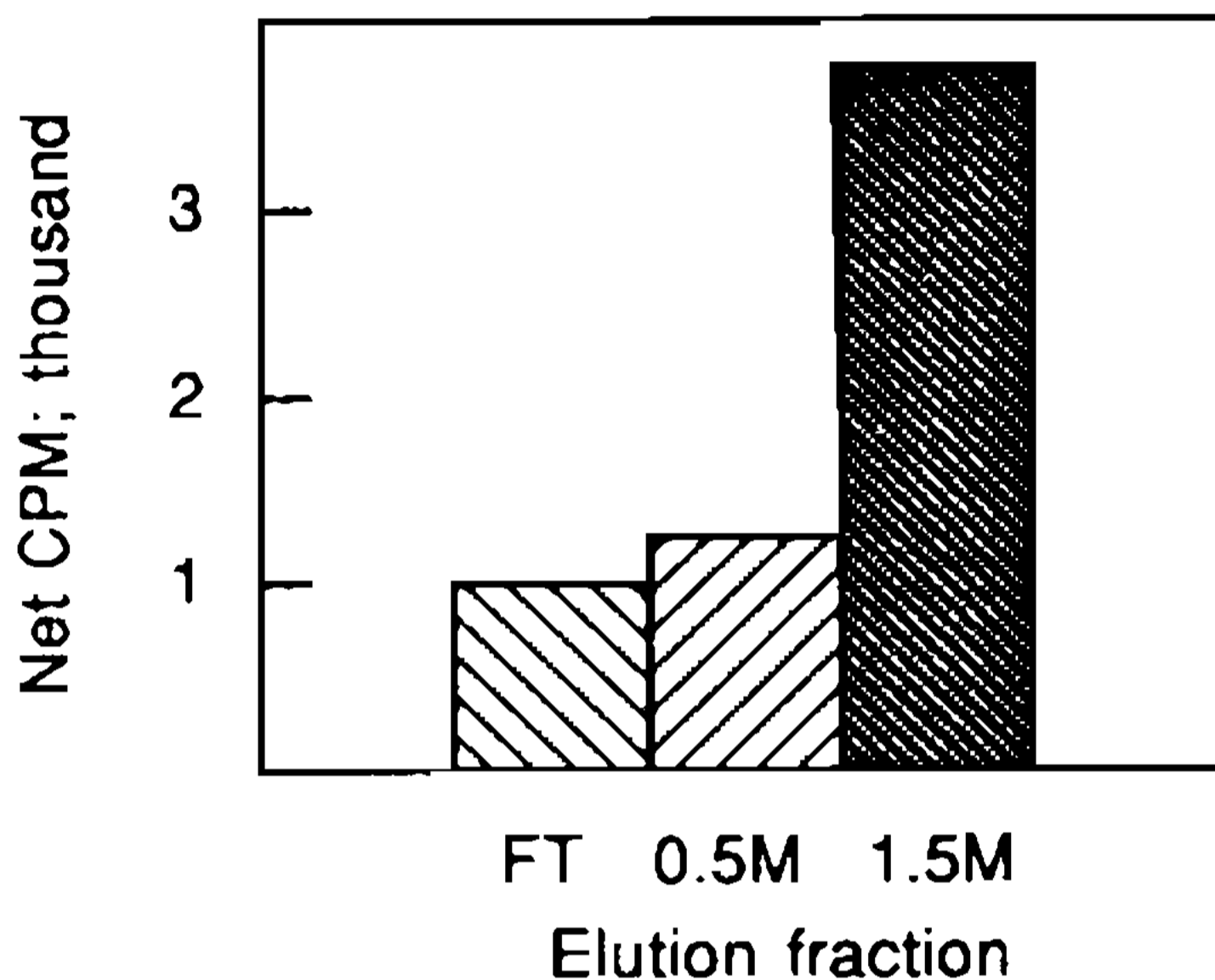

Fig. 3: fibroblast DNA synthesis (3_H-thymidine incorporation) in response to granuloma supernatant fractionated by heparin-Sepharose affinity chromatography. Neither the fraction that did not bind to heparin-Sepharose (fall-through, FT) nor the fraction that eluted with 0.5M NaCl contained fibroblast growth-stimulating activity. In contrast, the fraction that eluted with 1.5M NaCl contained a potent fibroblast mitogen.

The granulomas also produce factors that stimulate extracellular matrix synthesis by fibroblasts. Crude and fractionated granuloma supernatants stimulate net collagen, fibronectin, and hyaluronan synthesis in fibroblasts (Wyler et al., 1987; Prakash et al., 1991), chick calvaria (collagen; Wyler et al., 1987), and in hepatic fat-storing cells (fibronectin; Wyler, D. J. & Rojkind, M.; unpublished data). Notably, net matrix synthesis that occurs in response to granulomas represents unit increase in cellular activity and is not a consequence of cellular proliferation; enhanced synthesis begins in vitro long before fibroblast cytokinesis occurs.

The presence of these fibrogenic activities in culture supernatants of granulomas, obtained from infected euthymic mice, contrasts with
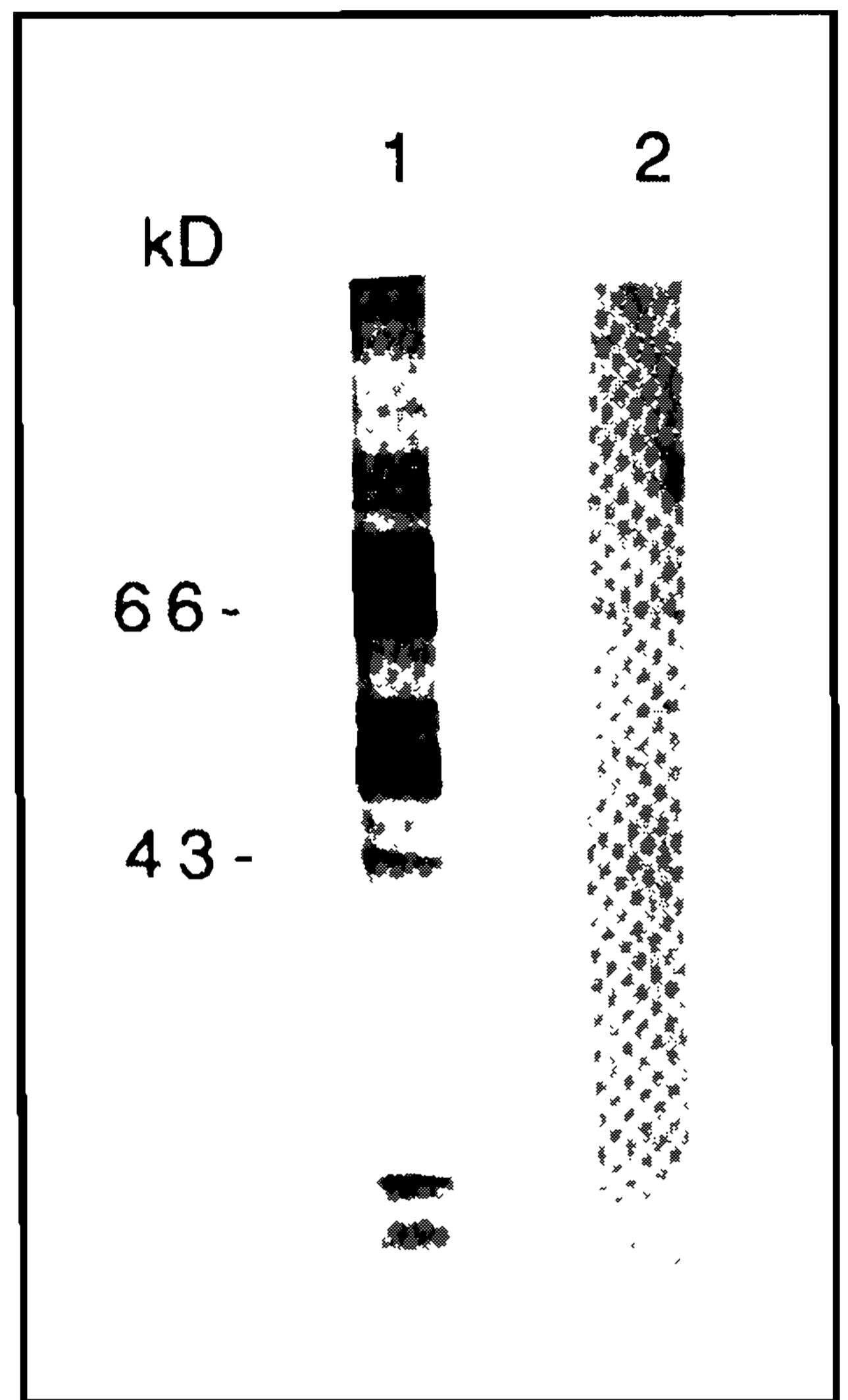

Fig. 4: detection with SDS-polyacrylamide gel electrophoresis of the purified heparin-binding fibroblast growth factor produced by egg granulomas. Unfractionated granuloma supernatant (lane 1) contains numerous silver-stainable proteins, whereas the 1.5M NaCl eluate from heparin-Sepharose (lane 2) contains only one detectable protein (Mr ≈60 kDA). The migration position of two molecular weight markers is shown.

their absence in supernatants of granulomas from similarly infected, congenitally athymic mice (Prakash et al., 1990).

Fibroblast stimulating factor-1 (FsF-1) is a novel growth factor produced by egg granulomas — The granuloma-derived fibroblast mitogen (FsF-1) has as one of its distinctive characteristics, avid binding to heparin-Sepharose. As such, it appears to be one of the so-called heparin-binding growth factors (Burgess & Maciag, 1989). The prototypic representatives of this family of relatively conserved proteins are acidic and basic fibroblast growth factors (FGF). However, FsF-1 is not detected by anti FGF antibody and antibody to the granuloma-derived cytokine (FsF-1) does not detect FGF. Furthermore, the amino acid content of FsF-1 and the FGFs are distinctive. The fact that FsF-1 is blocked at the amino terminus has prevented amino-terminal sequence analysis of

amino acids. An internal peptide (7 residues) of FsF-1 has been identified, the sequence of which has not been previously archived in a peptide databank. This observation, as well as experiments that indicate that FsF-1 is distinct from a variety of other cytokines, provides compelling evidence that FsF-1 is novel growth factor. Molecular cloning and sequencing indicate FsF-1 is unique.

FsF-1 is a lymphokine, probably secreted in response to egg antigens – Using anti FsF-1 antibody produced in rabbits, it is possible to identify the cellular source of FsF-1. Granulomas can be dissociated by collagenase treatment and monodispersed cell suspensions can be prepared. Cells can then be treated with antibody to FsF-1, followed by fluorescinated anti rabbit IgG, and then with phycoerythrin-stained anti CD4 antibody. When the population is then subjected to flow fluorocytometry, it is observed that twenty to 25 percent of the cells that stain with anti CD4 ($CD4^+$ lymphocytes) also stain with the anti FsF-1 antibody.

The suggestion from these studies that FsF-1 is a product of $CD4^+$ lymphocytes is corroborated by the following. Granuloma cells are stained with phycoerythrin-conjugated anti CD4 antibody and subjected to fluorescence-activated cell sorting. The $CD4^+$ cells are then cultured briefly in ^{35}S-methionine/cysteine-containing medium or non-radioactive medium. The culture supernatants (respectively) are subjected to immunoprecipitation with anti FsF-1 antibody followed by SDS-polyacrylamide gel electrophoresis, and to biological assay. Although several labelled peptides of various sizes are detected in the lymphocyte supernatants, immunoprecipitation with anti FsF-1 yields only one metabolically-labelled protein (Mr 60kDa). The migration of this protein corresponds exactly with the granuloma supernatant protein purified by heparin-affinity chromatography (Prakash & Wyler, 1991) and that recognized as FsF-1 in granuloma supernatants subjected to Western blot. Normal (pre-immune) rabbit Ig detects no proteins. The $CD4^+$ lymphocyte supernatant stimulates fibroblast proliferation.

The observations indicate that some of the $CD4^+$ lymphocytes isolated from egg granulomas produce FsF-1 spontaneously and also express FsF-1 on their membranes. It remains to be determined whether the secreting cells and the cells expressing surface FsF-1 are the

same (suggesting membrane anchoring, as shown for some other growth-promoting cytokines [Massague, 1990]), or whether selected lymphocytes have receptors for FsF-1 and bind the protein produced by other lymphocytes.

Efforts are under way to determine what triggers FsF-1 production by lymphocytes in the granuloma. Indirect evidence suggests that FsF-1 secretion occurs in response to egg antigens. Partially-purified soluble egg antigen can stimulate production of a fibroblast mitogen by splenocytes from schistosome-infected mice (Wyler & Tracy, 1982). The fact that $CD4^+$ lymphocytes isolated from granulomas spontaneously secrete FsF-1 *in vitro*, without addition of exogenous stimuli to culture, suggests that these cells were stimulated *in vivo* to secrete the growth factor.

Chronic infection results in the down-regulation of production of FsF-1 and other fibrogenic cytokines – It has been established previously that extracellular matrix synthesis and deposition in liver is reduced during the chronic phase of schistosome-infection in inbred mice (el Meneza et al., 1989). This observation afforded an excellent opportunity to test the hypothesis that there is a causal relationship between fibrogenic cytokine production in the granuloma and fibrogenesis in the liver.

A large group of mice were infected on the same day and subpopulations were sacrificed at intervals thereafter. Granulomas were isolated, cultured by routine methods, and cell-free supernatants were assayed for relevant biological activity (Prakash et al., 1991). Supernatants representing mice early in infection were the most active in stimulating fibroblasts to replicate and produce extracellular matrix (Fig. 5). In contrast, supernatants from granulomas obtained relatively late in infection were deficient in these activities. Since the differences were observed with supernatants tested over a wide range of dilutions, they reflect unambiguous distinctions in granuloma function.

Since oviposition occurs throughout infection, at any specific point in time the hepatic egg granulomas comprise a heterogeneous population. The changes observed in fibrogenic cytokine production in chronic infection could therefore represent involution of granulomas
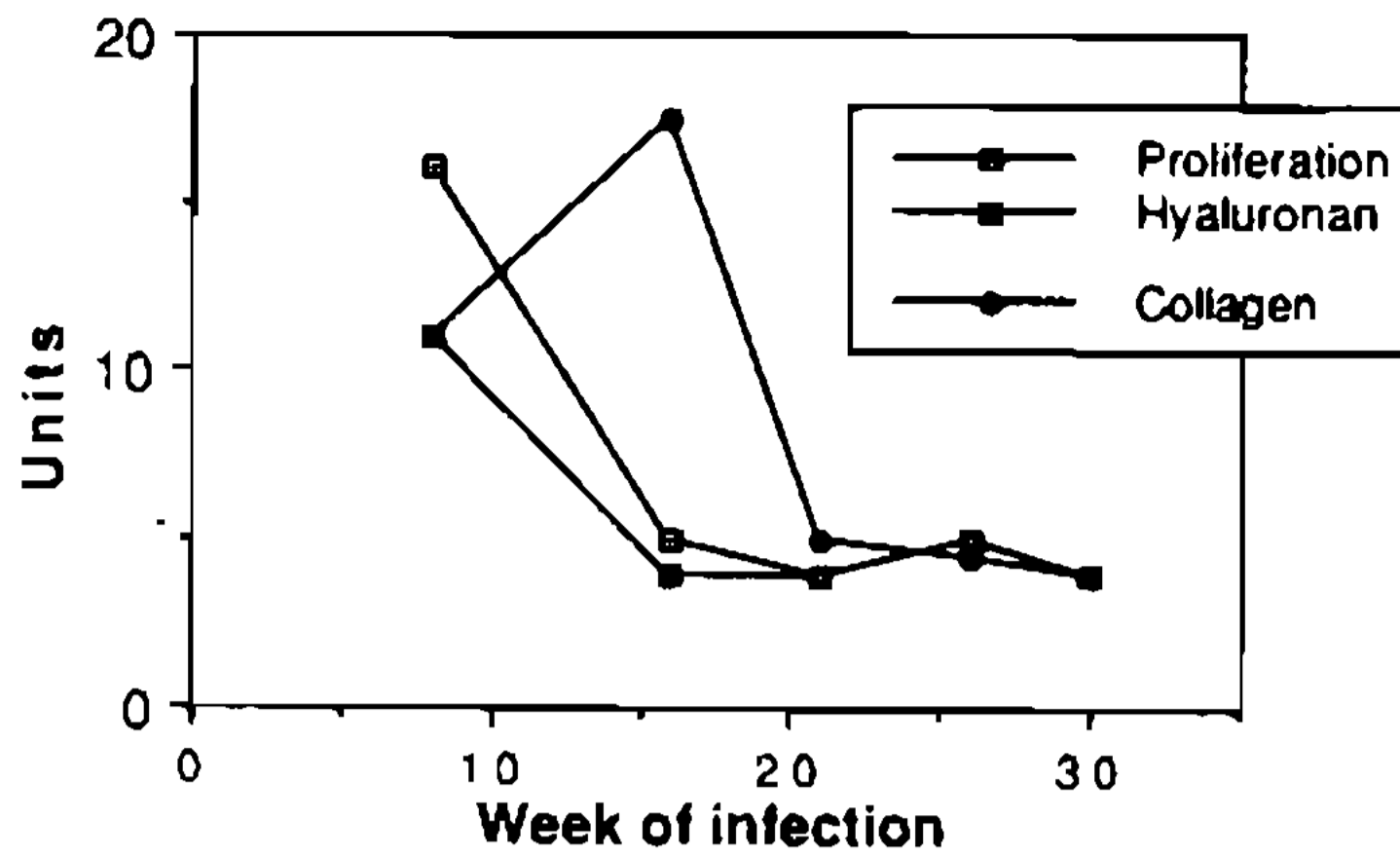
Fig. 5: alteration during the course of *Schistosoma mansoni* infection in the production by granulomas of proteins with different fibrogenic activities. Granulomas were isolated from liver of mice infected for the times indicated, incubated in serum-free medium, and cell-free culture supernatants were then added to fibroblast cultures. The net fibroblast proliferative response, and net synthesis of collagen and hyaluronan, induced by these granuloma supernatants was compared. The responses are indicated as relative units to permit comparisons of the three parameters.
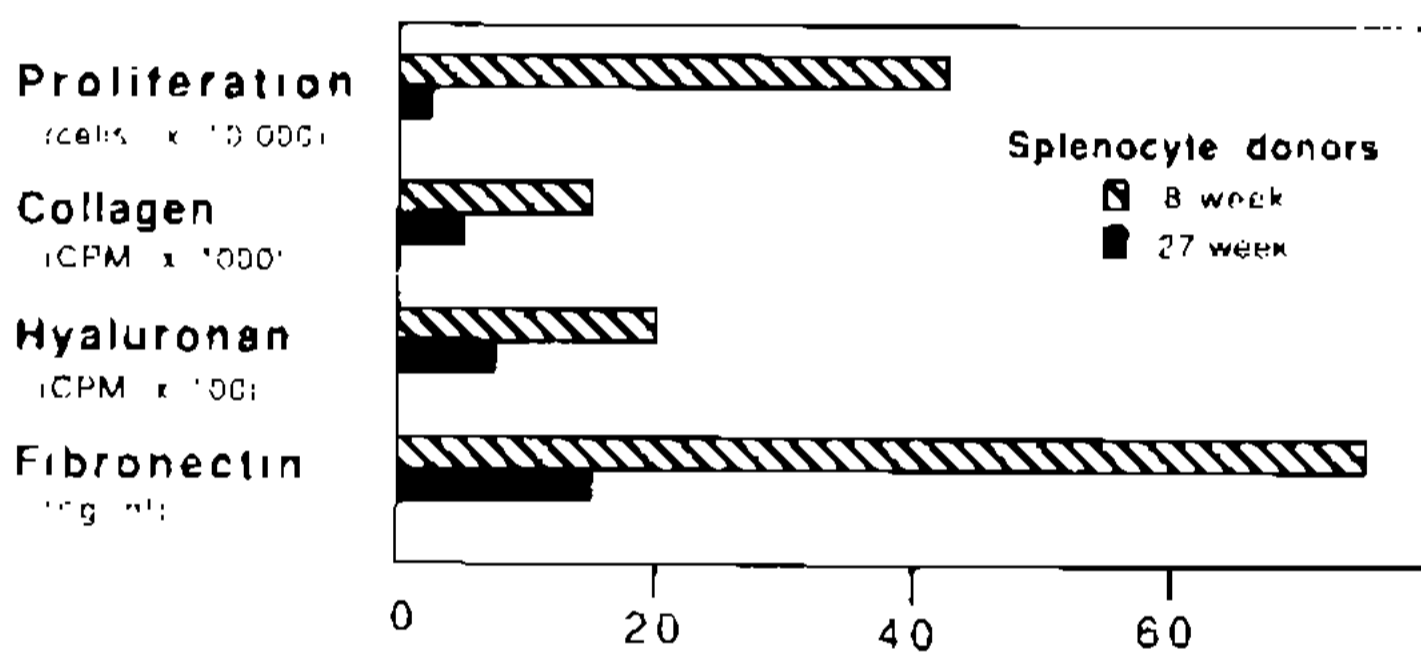

Fig. 6: response of fibroblasts to cell-free supernatant from granulomas obtained from mice at 8 weeks of infection with *Schistosoma mansoni* that at week 4 had received splenocytes from either mice infected for 8 weeks or for 27 weeks. Mean response elicited by supernatants of granulomas from 3-4 mice is shown; SEM ≤ 15%.

formed early in infection, or be a characteristic of those produced late in infection. To distinguish these possibilities, splenocytes from acutely-infected and from chronically-infected mice were adoptively transferred to infected recipients prior to the onset of oviposition. Hepatic granulomas were then retrieved from the recipients at 8 weeks of infection, when maximum fibrogenic activity in granuloma culture supernatants could be expected (Fig. 5). The recipients of splenocytes from acutely-infected mice produced hepatic granulomas whose culture supernatants actively stimulated a variety of fibroblast responses (Fig. 6). The magnitude of these responses was similar to those elicited by products of granulomas from mice infected for 8 weeks that were not in-

fused with any donor splenocytes. In dramatic contrast, the culture supernatants of granulomas isolated from liver of mice given splenocytes from chronically-infected mice lacked most of the fibrogenic activity (Fig. 6). Thus, it appears that the quality of the granulomas produced in response to egg deposition, rather than their involution with time, determines whether they produce fibrogenic cytokines. Indeed, the adoptive transfer studies provide compelling preliminary evidence that the regulation of fibrogenic cytokine production by hepatic granulomas is under immunological control.

DISCUSSION

These findings indicate that hepatic egg granulomas produce a variety of fibrogenic cytokines, including an apparently novel lymphokine, FsF-1. Furthermore, they support the conclusion that there is a causal relationship between the elaboration of the cytokines and hepatic fibrogenesis. First, congenitally athymic mice infected with *S. mansoni* produce small, lymphocyte- and eosinophil-depleted granulomas that produce little fibrogenic activity and do not develop liver fibrosis. Second, during chronic infection, fibrogenic cytokine production and fibrogenesis spontaneously undergo down-regulation. The fact that adoptive transfer of splenocytes from chronically-infected mice can abrogate both cytokine production (Fig. 6) and fibrogenesis (Olds et al., 1989) is particularly compelling evidence in support of a causal relationship.

The results of studies in mice have implications for understanding the pathogenesis of severe liver fibrosis in human schistosomiasis mansoni infection. Their relevance takes into account the following considerations. We recognize granulomatous inflammation in this disease as a consequence of a cascade of immunological events triggered by specific responses of sensitized T lymphocytes to egg antigens (Colley 1987). (The studies summarized here add the production of fibrogenic cytokines as among these responses). Down-regulation of the T cell responses (by immunological pathways currently under intensive investigation) is a predictable feature of chronic infection in mice and appears to occur in some patients (Colley et al., 1986). In this analysis, in vitro lymphoproliferative responses to egg antigens have been assessed in schistosomiasis patients as a presumptive surrogate marker of

the relevant proinflammatory responses and of their down-regulation during various stages of infection.

A provocative set of observations indicate that, in contrast to infected inbred strains of mice, not all patients undergo spontaneous down-regulation of the anti helminth antigen T cell responses (Colley et al., 1986). Indeed, when the chronic schistosomiasis patient study populations are stratified according to the presence of liver fibrosis (assessed by clinical [Colley et al., 1986] or ultrasonographic [Hafez et al., 1991] criteria), striking differences in T cell responses to parasite antigens are observed. The patients without evidence of liver fibrosis mount weak in vitro proliferative responses to schistosomal antigens, reflecting spontaneous down-regulation of those vigorous responses that are detectable early in infection. In contrast, most of the patients with liver fibrosis mount vigorous in vitro responses, suggesting a defect in these patients' ability to down-regulate antischistosomal hypersensitivity.

Why might this be so? One possibility is that persistence of T cell responsiveness plays a pathogenic role in hepatic fibrogenesis. Having determined that granuloma T cells produce FsF-1, a potent fibroblast mitogen, (Prakash & Wyler, 1991), and that egg antigens can trigger fibroblast mitogenic cytokine production (Wyler & Tracy, 1982), the relationship between specific T cell activation and fibrogenesis is apparent. Although inbred mice infected with S. mansoni develop many of the same histological features of liver fibrosis as infected humans, they do not develop grossly-demonstrable Symmer's fibrosis (Andrade, 1987). It appears, therefore, that the acute production of fibrogenic cytokines early in infection (Fig. 5) is insufficient to induce marked periportal fibrosis. Indeed, since hepatic fibrogenesis in mice virtually ceases when fibrogenic cytokines are no longer produced late in infection (el Meneza et al., 1989; Prakash et al., 1991), it stands to reason that persistent production of the fibrogenic cytokines is required for the development of extensive fibrosis.

This hypothesis leaves open the crucial question, what determines wheter an individual will experience spontaneous down-regulation of the parasite-driven T cell responses? The answer might lie at the level of genetic restriction of antigen response (or non-response). A carefully conducted study from Egypt con-

cluded that both the persistence of vigorous in vitro T cell responsiveness to helminth antigens and the development of hepatic fibrosis in schistosomiasis mansoni patients were linked to the MHC haplotypes A2 and B12, and the presence of fibrosis and vigorous T cell responsiveness was inversely correlated with the DR2 haplotype (Hafez et al., 1991). The suggestion that failure to down-regulate the T cell responses is genetically determined, and that this selection influences hepatic fibrogenesis, could explain why inbred mice (who lack the appropriate MHC alleles) uniformly down-regulate their anti-helminthic T cell responses and fail to develop the equivalent of Symmer's fibrosis (Fig. 7).

In concluding, it is tempting to speculate on how knowledge of the pathogenesis of liver fibrosis might ultimately have clinical utility. The fact that not all patients with liver fibrosis reverse this pathology following antihelminthic chemotherapy (for example see Homeida et al., 1988), and that some patients in fact upgrade the magnitude of fibrosis after treatment, suggests that antihelminthic chemotherapy may be insufficient in disease control for some patients. Drugs with antifibrotic activity (such as colchicine [Mansour et al., 1988; Brenner & Alcorn, 1990]) might be useful adjuncts in the patients inadequately managed with antihelminthic therapy alone. As we learn more about the molecular aspects of fibrogenesis, and particularly the identify of the fibrogenic cytokines, the possibility will exist of designing drugs that interfere with the cytokine activity.

A more global and ambitious perspective of the clinical relevance of this line of investigation relates to the fact that only a small subpopulation of infected individuals develop the clinically important complications of liver fibrosis (Chen & Mott, 1988). If it were possible to identify relatively early in infection those individuals at risk for developing such complications, a new strategy of disease control could be considered. Only those individuals at risk for disease might be the targets of available and projected control modalities (vaccination, antihelminthic chemotherapy and, if necessary, antifibrotic drugs). One could argue that those individuals infected but at diminishingly low risk for developing disease would be expected to benefit little from immunoprophylaxis or chemotherapy. Therefore, specifically identifying and targeting those at risk

of illness should favorably influence the cost-benefit relationship of schistosomiasis disease control efforts. While our current state of knowledge precludes testing such an approach in the near future, it seems timely that the array of molecular and genetic technologies currently available be more vigorously applied to defining the basis of disease risk in schistosomiasis. Balancing the current enthusiasm for antischistosomal vaccine development with a redoubled effort at gaining a better understanding of the pathogenesis of liver fibrosis may pay off in the long run.

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