

Molecular Characterization of an Interleukin-4-inducing Factor from *Schistosoma mansoni* Eggs*

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The eggs of the parasitic trematode *Schistosoma mansoni* are powerful inducers of a T helper type 2 (Th2) immune response and immunoglobulin E (IgE) production. *S. mansoni* egg extract (SmEA) stimulates human basophils to rapidly release large amounts of interleukin (IL)-4, the key promoter of a Th2 response. Here we show purification and sequence of the IL-4-inducing principle of *S. mansoni* eggs (IPSE). Stimulation studies with human basophils using SmEA fractions and natural and recombinant IPSE as well as neutralization and immunodepletion studies using antibodies to recombinant IPSE demonstrate that IPSE is the bioactive principle in SmEA leading to activation of basophils and to expression of IL-4 and IL-13. Regarding the mechanism of action, blot analysis showed that IPSE is an IgE-binding factor, suggesting that it becomes effective via cross-linking receptor-bound IgE on basophils. Immunohistology revealed that IPSE is enriched in and secreted from the subshell area of the schistosome egg. We conclude from these data that IPSE may be an important parasite-derived component for skewing the immune response toward Th2.

Infection with the parasitic trematode *Schistosoma mansoni* leads to a pronounced Th2¹ response and to elevated IgE production both in humans and in experimental animals. The definition of parasite-derived products capable of skewing the immune response toward Th2 would not only enhance our understanding of the defense mechanisms involved in helminth infections but may also lead to new insights into the pathogen-

esis of immediate-type hypersensitivity diseases such as asthma. However, in contrast to our increasing understanding of how pathogen-derived products can initiate Th1-type immune responses, there is so far little detailed knowledge about the nature of the parasite-derived molecule(s) and the underlying mechanisms that trigger and/or amplify a Th2-type reaction. In *S. mansoni* infection, a critical role in inducing a polarized Th2 response is played by the egg stage of the parasite (1), since a Th2 response and IgE production are only observed after egg deposition or following injection of schistosome eggs (2) or extracts thereof (3) into naive animals. By contrast, the initial larval (schistosomula) and adult worm stages rather induce a response skewed to Th1.

It is now firmly established, both *in vivo* and *in vitro*, that the cytokine profile present during an immune reaction is an important element in directing the response to Th1 or Th2 and that IL-4 is the key cytokine responsible for biasing the immune reaction toward a Th2 phenotype (4–7). In the human system, basophils are a prominent source of IL-4 and IL-13; these cells secrete large amounts of IL-4 and IL-13 in response to IgE-receptor cross-linking or activation by a combination of IL-3 and C5a (8, 9). Indeed, human basophils can be viewed as “innate Th2-type” effector cells, since IL-4 and IL-13 are expressed in a very restricted manner without production of any of the cytokines involved in Th1-type immune responses. We therefore wondered whether saline-soluble *S. mansoni* egg antigen extract (SmEA) can directly trigger human blood basophils to release IL-4, thus providing a potential mechanism for biasing the immune response to Th2 during the egg stage of *S. mansoni* infestation. Indeed, our previous studies showed that basophils from healthy nonsensitized donors from Northern Europe rapidly degranulate and release histamine, sulfido-leukotrienes, and considerable amounts of IL-4 as well as IL-13 in response to exposure to SmEA (10) but not to extracts of schistosomula.² SmEA thus obviously contains a basophil-degranulating IL-4-inducing principle, prompting us to further characterize this activity. Subsequent studies revealed that the active principle is an excretory/secretory glycoprotein interacting with IgE on basophils in an antigen-nonspecific way (11).

Here we present the isolation, sequencing, and cloning of the bioactive product, which was named IL-4-inducing principle from *S. mansoni* eggs (IPSE). Finally, as unambiguous proof of the identity of the cloned *S. mansoni* product, we demonstrate that the recombinant protein has full biological activity.

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¹ The abbreviations used are: Th2, T helper type 2; IL, interleukin; aa, amino acid(s); AAA, *A. aurantia* agglutinin; EST, expressed sequence tag; Fcε RI, high affinity IgE receptor; IPSE, interleukin-4-inducing principle from *S. mansoni* eggs; PBS, phosphate-buffered saline; SmEA, saline-soluble *S. mansoni* egg antigen extract; SmE-library, *S. mansoni* egg cDNA library; Dig, digoxigenin; AP, alkaline phosphatase; RANTES, regulated on activation normal T cell expressed and secreted.

² T. Brunner and C. A. Dahinden, unpublished data.

EXPERIMENTAL PROCEDURES

Antigens

S. mansoni eggs were purified as previously described (12). SmEA was prepared by the methods of Boros and Warren (13) and Carter and Colley (14) and was obtained from Dr. F. Yousif (Schistosome Biological Supply Program, Theodor Bilharz Research Institute, Imbaba, Egypt).

Purification, Culture, and Stimulation of Basophils

Human basophils were purified by a previously described three-step procedure (15) and used at a mean purity of 99%. Purified basophils were incubated in round-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) at 37 °C in humidified air containing 6% CO₂ at 10⁶ basophils/ml in a final volume of 100 µl of culture medium (Iscove's modified Dulbecco's medium supplemented with 100 units/ml penicillin G, 50 µg/ml transferrin, 5 µg/ml insulin, 100 µg/ml streptomycin, 10% fetal calf serum). Since IL-3 enhances Fcε RI-mediated (16) and SmEA-mediated (10) IL-4 production of basophils, all wells were supplemented with IL-3 except the wells to which ionomycin was added. The cells were incubated with various stimuli at the concentrations indicated. If not stated otherwise, the supernatants were collected after 18 h and stored at -20 °C until further investigation. Degranulation of basophils was assessed via cytospin preparation and May-Gruenwald staining.

Cytokine and Histamine Assays

IL-4 and IL-13 levels in culture supernatants were determined using two-site sandwich enzyme-linked immunosorbent assays (Eli-Pair; Diaclone, Besancon, France) essentially according to the manufacturer's protocol. The sensitivities for IL-4 and IL-13 detection were 0.55 and 1.56 pg/ml, respectively. Histamine was determined using the methyl-histamine radioimmune assay (Amersham Biosciences) according to the manufacturer's recommendations.

SDS-PAGE and Blotting

Proteins were separated by SDS-PAGE (12% T, 4% C) under reducing and nonreducing conditions according to Laemmli (17). For protein detection on the gels, silver staining was performed (18). For blotting analysis, separated proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell) via semidry blotting (30 min, 0.8 mA/cm²) (19). Free binding sites were blocked by incubating the membrane with 0.05% (v/v) Tween 20 in 0.1 M Tris-buffered saline, pH 7.4. The membranes were then incubated with alkaline phosphatase-labeled *Aleuria aurantia* agglutinin (AAA; 1:5000; Vector Laboratories, Burlingame, CA) or with antibodies (at the concentrations indicated) in 0.1 M Tris-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20. Lectin and antibody binding was visualized by a substrate/chromogen mixture of 0.033% (w/v) nitro blue tetrazolium and 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (Serva, Heidelberg, Germany) in 0.1 M Tris-buffered saline, pH 9.5 (20).

Purification of IPSE

IPSE was purified by cation exchange and affinity chromatography. First, 2 mg of SmEA in 20 mM potassium phosphate buffer, pH 5.0, were bound to a HiTrap SP-Sepharose column (1 ml; Amersham Biosciences). Elution of bound proteins was carried out using a linear salt gradient from 0 to 1 M KCl. Effluent and eluate fractions were analyzed for their IL-4-inducing effect (applied volume: 5 µl/10⁶ basophils) and (after speedvac concentration) for their protein banding pattern. The latter was determined by SDS-PAGE followed by silver staining. Fractions containing IPSE were pooled and concentrated, and for affinity chromatography, the buffer was exchanged to 50 mM sodium phosphate buffer, pH 7.0, by using Centricon Plus-20 centrifugal filters (Millipore Corp., Bedford, MA).

For affinity chromatography, AAA (2 mg; Vector Laboratories) was coupled to *N*-hydroxysuccinimide-activated Sepharose HiTrap columns (Amersham Biosciences) according to the manufacturer's instructions. After application of pooled IPSE-containing fractions and washing, bound material was eluted with 0.1 M L-fucose in 50 mM sodium phosphate buffer, pH 7.0. Fractions containing IPSE were pooled, concentrated by Centricon Plus-20 centrifugal filters, and stored at -80 °C.

N-terminal Sequencing of Purified IPSE

N-terminal sequencing of the first 20 aa of the purified IPSE was performed on an Procise 492 Sequencer (Applied Biosystems) at Chromatec Ltd. (Greifswald, Germany).

S. mansoni Egg cDNA Libraries (SmE-libraries)

SmE-library A—mRNA was isolated from 1 × 10⁶ *S. mansoni* eggs using the µMACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. 3 µg of mRNA were obtained and used for construction of a cDNA library in λ ZAP II by means of the Lambda ZAP II XR library construction kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The resulting cDNA library of *S. mansoni* eggs had 70% inserts and a titer of 6 × 10⁹ plaque-forming units/ml.

SmE-library B—The other SmE-library was constructed as previously described (21). This library has been extensively used for the generation of egg stage expressed sequence tags (ESTs) (22).

Screening of SmE-libraries Using a Dig-labeled Oligonucleotide

For screening of the cDNA libraries, a sequence-specific 30-mer oligonucleotide (for sequence, see Fig. 3) was labeled with Dig-ddUTP using the Dig oligonucleotide 3'-end labeling kit (Roche Diagnostics). The labeled oligonucleotide was detected via an AP-conjugated anti-Dig antibody (1:5000; Roche Diagnostics). Plaque lifts and hybridization were performed as described by Sambrook and Russell (23). Hybridization was carried out overnight at 55 °C in 5× SSC, 1% (w/v) blocking reagent (Roche Diagnostics), 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS. Prior to detection of bound oligonucleotide, filters were washed twice for 5 min at room temperature with 2× SSC, 0.1% (w/v) SDS and twice for 15 min at 55 °C with 0.2× SSC, 0.1% (w/v) SDS. Positive phage clones were isolated, and the pBluescript SK plasmids containing the inserts were obtained by *in vivo* excision using the helper phage Exassist (Stratagene). Automated sequencing of double-stranded plasmid DNA was performed by cycle sequencing using the Big Dye Terminator Cycle Sequencing ready Reaction Kit on an ABI 377 DNA sequencer (PerkinElmer Life Sciences).

Expression and Purification of Recombinant IPSE in *Escherichia coli*

Recombinant IPSE was expressed as a His tag fusion protein (His-IPSE) in *E. coli*. cDNA coding for IPSE was subcloned into the pProEX HTb expression vector (Invitrogen) coding for 6 N-terminally located histidine residues and a cleavage site for TEV protease. For this purpose, PCR was performed with IPSE-specific primers containing an AT clamp and the following restriction sites (underlined): *EheI* for the sense primer (5'-ATATATGGCGCCGATTTCATGCAAAATATTGTC-3') and *HindIII* for the antisense primer (5'-ATATATAAGCTTTTCATCAGTTCATATGC-3'), respectively. Conditions for PCR were as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 44 °C, and extension for 1 min at 72 °C (30 cycles). Subcloning and confirmation of DNA sequences followed standard procedures (23). *E. coli* cultures containing the recombinant plasmid were grown at 37 °C to an A₆₀₀ of 0.8, and then expression of His-IPSE was induced by 0.6 mM isopropyl thioalgalactoside. After incubation for an additional 4 h, cells were harvested by centrifugation and resuspended in 30 ml 50 mM Tris/HCl, pH 8.0, 0.8 mM EDTA, 200 mM NaCl, 25% sucrose. His-IPSE was recovered as insoluble inclusion bodies after breaking the cells by passing them twice through a French press (at a gauge reading of 1000 p.s.i.) and subsequent centrifugation at 7000 × *g*. The insoluble fraction was washed once with 20 mM Tris/HCl, pH 8.0, 0.5% deoxycholate and once with 20 mM Tris/HCl, pH 8.0, and subsequently solubilized in 8 M urea, 500 mM NaCl, 50 mM sodium phosphate, pH 7.5. His-IPSE was purified by nickel-nitrilotriacetic acid metal affinity chromatography (Qiagen, Hilden, Germany) under denaturing conditions according to the manufacturer's protocol. Using the Hampton Research FoldIT Screen (Hampton Research, Laguna Niguel, CA), optimal buffer conditions for refolding of the denatured His-IPSE were obtained. Briefly, purified His-IPSE was refolded by dialysis at a concentration of 0.1 mg/ml against FoldIT buffer 3 (modified to pH 10.5) and subsequently against a physiological buffer such as phosphate-buffered saline (PBS).

Preparation of Monoclonal Antibodies and Murine Antiserum to IPSE

Monoclonal antibodies and murine antiserum to His-IPSE were raised by Labsoft Diagnostics AG (Halle, Germany). To obtain monoclonal antibodies, aliquots containing 28 µg of His-IPSE emulsified in 100 µl of saline (154 mM) and 300 µl of complete Freund's adjuvant were intraperitoneally administered to 10-week-old female BALB/c mice. The intraperitoneal immunization was repeated after 9 weeks (28 µg of antigen with incomplete adjuvant). The third (after further 2 weeks) and the fourth injection (3 days thereafter) of IPSE (each 28 µg) were administered in saline (murine antiserum to IPSE was raised by a

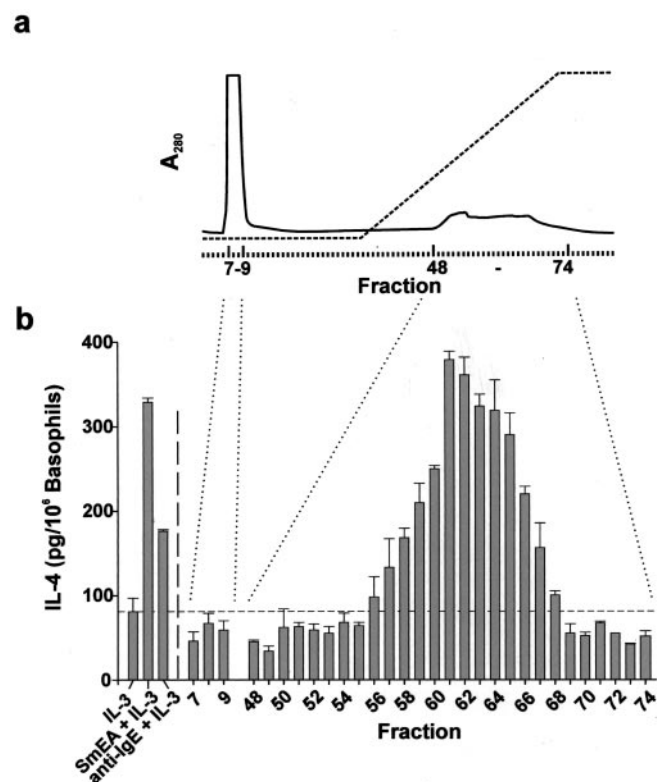


FIG. 1. Purification of IPSE by cation exchange chromatography. *a*, A_{280} light absorption by fractions of SmEA after cation exchange chromatography on SP-Sepharose. Adherent molecules were eluted with a continuous gradient of 0–1 M KCl. Solid line, A_{280} ; dashed line, salt gradient. *b*, IL-4 production by basophils stimulated with the SmEA fractions obtained by chromatography on SP-Sepharose in *a*. Histogram bars indicate the amount of IL-4 produced by purified basophils cultured with IL-3 alone (10 ng/ml), IL-3 and SmEA (10 μ g/ml) or anti-IgE (100 ng/ml), or IL-3 and a 5 μ l/ml concentration of each SP-Sepharose fraction.

similar immunization protocol). One day after the last booster injection, the animals were killed and blood was taken for antiserum preparation. The fusion was performed according to a standard procedure using the mouse nonsecretory cell line P3X63Ag8.653 as fusion partner.

Immunohistological Detection of IPSE

Liver sections (formalin-fixed, paraffin-embedded material) of mice infected with 150 *S. mansoni* cercariae 7 weeks previously were incubated overnight with the monoclonal anti-IPSE antibody 74 1G2 (undiluted culture supernatant) or (as negative controls) with murine IgG (100 μ g/ml in PBS; Dianova, Hamburg, Germany) or PBS only. Subsequently, the sections were incubated for 1 h with AP-conjugated F(ab')₂ goat anti-mouse IgG antibodies (1:100; Dianova), followed by staining with Neufuchsin (Dako, Hamburg, Germany) and hematoxylin (Merck). All steps were carried out according to routine protocols.

Computer Analysis and Nucleotide Sequence Accession Number

Sequence data were analyzed with DNASIS software (Amersham Biosciences). The GenBank™ data base (NCBI) was searched for homologous sequences using the nucleotide and protein BLAST programs. Secondary structure predictions were performed online using the "Protein Predict server" available on the World Wide Web at maple.bioc.columbia.edu/predictprotein. The nucleotide sequence of IPSE was submitted to the GenBank™ data base and has been assigned GenBank™ accession number AY028436.

RESULTS

Fractionation of SmEA by Cation Exchange Chromatography—The optimal pH for binding the IL-4-inducing activity to the cation exchange matrix (SP-Sepharose) was determined in pilot experiments (not shown). At pH 5.0, nearly all activity was retained by the matrix, whereas the majority of irrelevant proteins were found in the effluent. A linear salt gradient (0–1

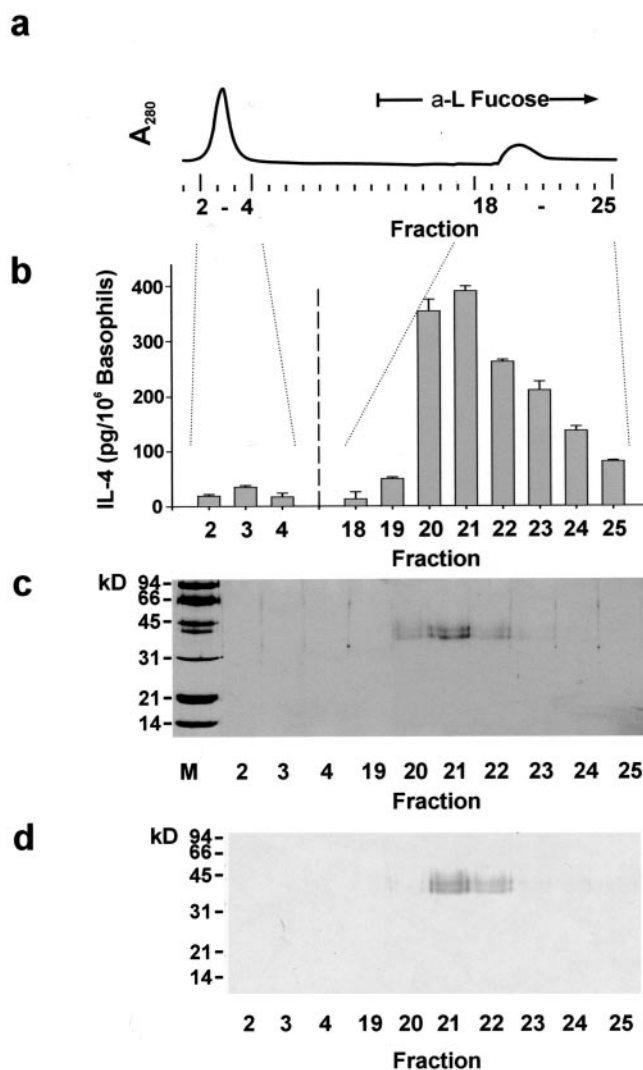


FIG. 2. Purification of IPSE using lectin chromatography. *a*, A_{280} protein trace of fractionation of pooled active SmEA fractions from SP-Sepharose on AAA-Sepharose. Adherent factors were eluted with 0.1 M L-fucose. *b*, IL-4 production by cultured basophils stimulated with effluent and eluate fractions (1 μ l/ml) from AAA-Sepharose in *a*. *c*, SDS-PAGE of eluate fractions from AAA-Sepharose followed by silver staining. *d*, SDS-PAGE of eluate fractions from AAA-Sepharose followed by blotting onto nitrocellulose membrane and incubation with labeled AAA.

M KCl) was employed for eluting the bound material. Under these conditions, the peak of the IL-4-inducing activity was found to elute within the eluate fractions representing only a minor proportion of the total protein (Fig. 1, *a* and *b*).

To assign the IL-4-inducing activity to a particular component, SDS-PAGE analysis and extended silver staining of speedvac-concentrated chromatographic fractions of SmEA were performed under nonreducing conditions, revealing a faint diffuse double band at 40 kDa that shifted to about 20 kDa under reducing conditions. The staining intensity of the 40-kDa double band correlated with the IL-4-inducing activity of the active fractions (not shown). Previous molecular mass determinations of the IL-4-inducing activity using electroelution of gel sections of SmEA separated by SDS-PAGE indicated a range between 31 and 66 kDa (11), further suggesting that the 40-kDa double band or one of its components might represent the bioactive principle.

The IL-4-inducing Principle Binds to *A. aurantia* Agglutinin—Earlier investigations had indicated that the active prin-

1	M	F	L	I	A	V	L	S	Y	T	L	I	S	Q	L	G	I	T	T	19
1	ATG	TTT	CTT	ATT	GCC	GTA	TTG	TCA	TAC	ACA	TTG	ATA	AGT	CAA	TTG	GGG	ATA	ACT	ACA	57
20	S	D	S	C	K	Y	C	L	Q	L	Y	D	E	T	Y	E	R	G	S	38
58	TCG	GAT	TCA	TGC	AAA	TAT	TGT	CTA	CAA	TTG	TAC	GAT	GAA	ACG	TAT	GAG	AGG	GGT	TCA	114
39	Y	I	E	V	Y	K	S	V	G	S	L	S	P	P	W	T	P	G	S	57
115	TAT	ATT	GAA	GTC	TAC	AAA	AGC	GTT	GGC	TCA	CTC	TCA	CCA	CCA	TGG	ACA	CCT	GGA	TCT	171
58	V	C	V	P	F	V	N	D	T	K	R	E	R	P	Y	W	Y	L	F	76
172	GTT	TGT	GTA	CCC	TTC	GTA	AAT	GAC	ACG	AAG	AGA	GAG	CGT	CCA	TAC	TGG	TAT	TTA	TTT	228
77	D	N	V	N	Y	T	G	R	I	T	G	L	G	H	G	T	C	I	D	95
229	GAC	AAC	GTC	AAT	TAC	ACA	GGT	CGG	ATT	ACT	GGT	CTC	GGA	CAT	GGT	ACC	TGC	ATT	GAT	285
96	D	F	T	K	S	G	F	K	G	I	S	S	I	K	R	C	I	Q	T	114
286	GAC	TTC	ACG	AAA	TCC	GGA	TTC	AAA	GGC	ATT	TCC	TCT	ATT	AAA	CGG	TGT	ATT	CAA	ACA	342
115	K	D	G	K	V	E	C	I	N	Q	P	K	R	R	R	T	Y	C	R	133
343	AAG	GAT	GGA	AAA	GTT	GAA	TGT	ATC	AAT	CAA	CCG	AAA	CGG	AGA	AGG	ACA	TAC	TGT	CGA	399
134	F	*																		134
400	TTC	TAA	TCA	AGA	AAT	GTA	ATG	CGC	TTA	TAA	TTC	TTG	TGT	GCT	CTT	AAC	TAA	ATA	TAT	456
457	GCA	TAT	GAA	CTG	ATG	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	489

FIG. 3. Nucleotide and predicted protein sequence of IPSE (GenBank™ accession number AY028436). The 489 nucleotides encode a 134-aa protein with a 20-aa signal peptide (**boldface type**). The N-terminal end of the mature protein, whose sequence was determined by protein sequencing, is **boxed in black**. From this sequence and the corresponding EST (AI820476) identified by a BLASTn search against dbEST, a 30-nt nondegenerated primer (indicated by an **arrow**) was designed and used as a probe for screening the SmE libraries. Also shown are the seven cysteine residues (**circled**) and the two potential N-glycosylation sites (**white boxes**); aa 73–89 (**boxed in gray**) correspond to a “Greek key” motif signature (PS00225) identified by ScanProsite. The polyadenylation signal is **underlined**.

ciple is a protein because of its sensitivity to protease digestion (11). Since schistosome extracts contain numerous glycosylated components (24), we asked whether the active principle is a glycoprotein and, thus, could be purified by lectin affinity chromatography. To address this point, pooled active fractions from cation exchange chromatography were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with a panel of nine labeled lectins with various glycan specificities. Indeed, two lectins bound to the 40-kDa double band (the putative active principle). One of them, AAA, seemed most suitable for affinity chromatography, since it reacted only with one additional irrelevant band of ~30 kDa that appeared in the late active fractions and was also present in the adjacent inactive fractions (data not shown). To exclude this irrelevant band, only the early active fractions were pooled for further purification. On AAA affinity chromatography, the effluent fractions were inactive, whereas the fractions eluted with 0.1 mM L-fucose contained IL-4-inducing activity (Fig. 2, a and b). SDS-PAGE followed by silver staining (Fig. 2c) or by blotting with labeled AAA (Fig. 2d) revealed that the eluate fractions contained only the double band that correlated in its intensity with the IL-4-inducing activity of the respective fractions. The eluate fractions did not contain other components detectable by staining, indicating a high purity of the isolated material. Thus, the active principle is most likely a glycoprotein of ~40 kDa containing α -linked fucose.

N-terminal Sequencing of IPSE—Attempts to separate the two bands at 40 kDa were not successful. Nevertheless, N-terminal sequencing of the pooled eluate fractions from AAA-Sepharose revealed an unequivocal sequence with minimal background for the first 20 N-terminal aa, suggesting that the double band represents different posttranslational forms of the same protein. Two aa in positions 3 and 6 could not be identified due to a low signal consistent with the presence of cysteines, as later confirmed by sequencing the IPSE cDNA (Fig. 4).

A similarity search did not reveal any sequences with significant homology in the public databases, with the exception of

one EST (NCBI nucleotide data base, NCBI accession number AI820476) from an *S. mansoni* egg cDNA library, sharing an overall similarity of 81% with the N-terminal sequence and 100% identity for aa 7–16.

Isolation of IPSE cDNA from *S. mansoni* Egg cDNA Libraries—To obtain the complete nucleotide sequence, two different *S. mansoni* egg cDNA libraries (SmE-library A and SmE-library B) were screened via plaque lift assay by hybridization. A nondegenerate 30-mer oligonucleotide spanning 10 homologous nucleotide triplets coding for aa 7–16 was chosen as a probe (Fig. 3). A total of 12 identical clones was isolated (six from each library). The complete sequence (Fig. 3) consisted of 489 nucleotides. Sequence analysis revealed an open reading frame of 405 nucleotides with a predicted coding capacity for 134 aa including a signal sequence of 20 aa. The mature protein of 114 aa had two putative N-glycosylation sites at aa 44 and 60, respectively, and seven cysteine residues. Importantly, all 20 aa of the predicted N terminus of the mature protein completely matched the aa sequence determined by N-terminal sequencing of the purified natural product. Upon screening the NCBI protein and nucleotide data banks, there was a sequence identity of 92% with the EST sequence mentioned above but no other significant similarities, indicating that related proteins have not yet been identified. The molecular mass of the active principle calculated from the sequence was 13.2 kDa, which is far below the apparent molecular mass of the unreduced purified double band at 40 kDa.

Expression of Recombinant IPSE—To confirm that IPSE cDNA encoded the active principle, recombinant IPSE was expressed in *E. coli* as a His tag fusion protein. His-IPSE was recovered from inclusion bodies under denaturing conditions in 8 M urea and purified via nickel-nitrilotriacetic acid metal affinity chromatography. The protein was refolded by dialysis against the FoldIT buffer 3 (Hampton Research) adjusted to pH 10.5 and then against PBS. Refolding was performed at a low protein concentration (0.1 mg/ml), because higher concentrations led to severe protein losses due to precipitation.

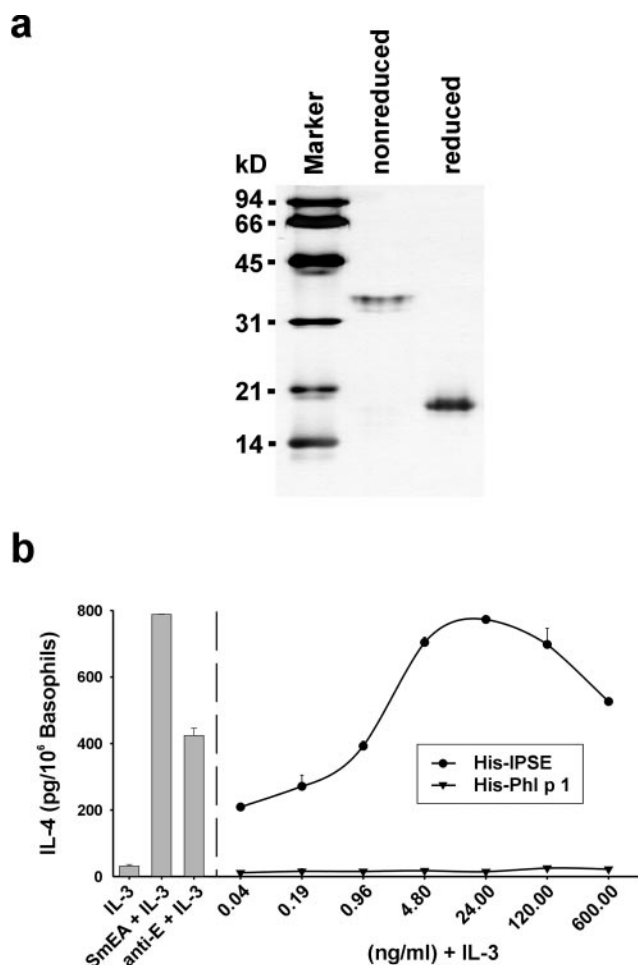


FIG. 4. Formation of homodimers and dose-dependent induction of IL-4 production in basophils by recombinant IPSE. *a*, SDS-PAGE and silver staining of His-IPSE under reduced and nonreduced conditions. *b*, IL-4 production by basophils stimulated with various amounts of His-IPSE or with a control His tag fusion protein (*His-Phl p1*), respectively.

As assessed by SDS-PAGE under nonreducing conditions, refolded His-IPSE formed dimers that could be reversed into monomers under reducing conditions (Fig. 4*a*). When tested on human basophils, His-IPSE had a pronounced IL-4-inducing activity (Fig. 4*b*) and was in this respect equivalent to its natural counterpart. The dose-response curve had its maximum at 24 ng/ml or 0.75 nM, respectively, and displayed a bell-shaped form that is similar to that of IgE-mediated basophil activation.

The identity of the recombinant protein with natural IPSE was further proven by the following experiments. A mouse antiserum raised against His-IPSE was strongly reactive with both recombinant and natural IPSE but showed no cross-reactivity to other components of SmEA (Fig. 5*a*). Moreover, when basophils were stimulated with SmEA or (data not shown) with His-IPSE, the addition of a murine antiserum against His-IPSE dose-dependently inhibited IL-4 production (Fig. 5*b*). Finally, immunodepletion using the IgG fraction of murine anti-His-IPSE antiserum (10 μ l) bound to protein G-Sepharose (4 Fast Flow; Amersham Biosciences; 100 μ l of gel slurry as obtained from the manufacturer) removed the IL-4-inducing capacity from SmEA (100 μ g). In contrast, the IgG fraction of normal mouse serum as a control had no effect (data not shown). Taken together, our data clearly demonstrate that the recombinant material corresponds to IPSE.

IPSE Is the Principal Bioactive Component of SmEA and a

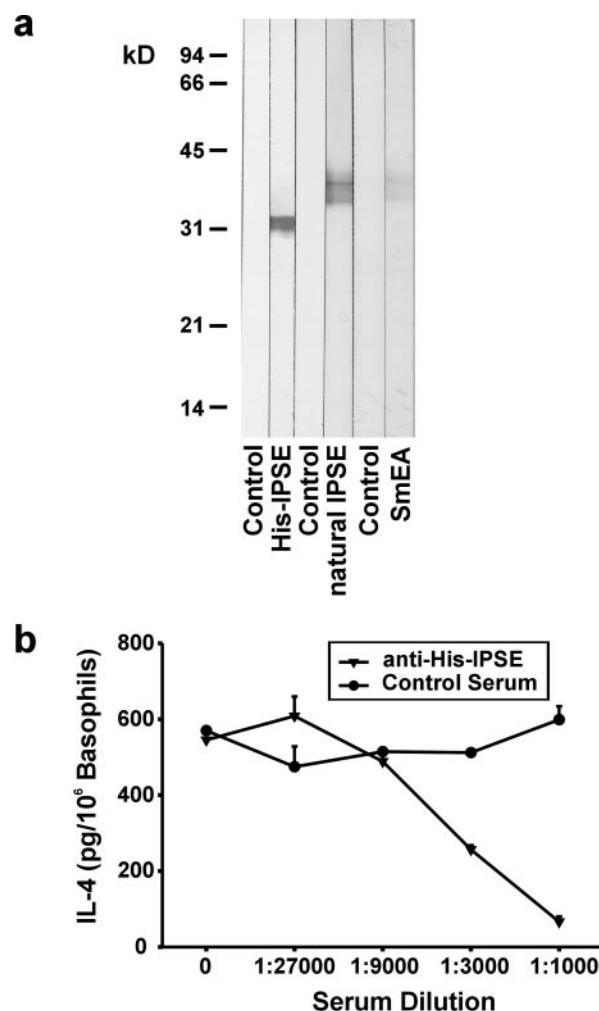


FIG. 5. Murine antiserum to recombinant IPSE reacts with and neutralizes natural IPSE. *a*, Western blots of His-IPSE, purified natural IPSE, and SmEA, respectively, were incubated with murine anti-His-IPSE antiserum (diluted 1:2000). Bound antibodies were detected with AP-labeled anti-murine IgG. Controls, binding of second antibodies to IPSE. *b*, anti-His-IPSE dose-dependently inhibits SmEA-induced IL-4 production. Human basophils were stimulated with SmEA (2 μ g/ml) in the presence of various dilutions of murine anti-His-IPSE antiserum. The culture supernatants were tested for IL-4 content (one representative out of two experiments).

General Activator of Human Basophils—SmEA leads to a general activation of basophils including degranulation and mediator release (10). To study whether all of these bioactivities are due to IPSE, basophils were incubated with whole SmEA, pooled SmEA fractions, and recombinant His-IPSE in parallel (Fig. 6). Pools 1 and 2, corresponding to effluent and early eluate fractions, respectively, from cation exchange chromatography, were devoid of IPSE. Pool 3, corresponding to the late eluate fractions, contained IPSE at a purity of at least 70%. Degranulation was observed when basophils were treated with SmEA anti-IgE, pool 3, or recombinant IPSE but not following incubation with pool 1 or 2. Degranulated basophils often formed considerable cell clusters, obviously due to agglutination. With regard to mediator release, pool 3 and recombinant IPSE, like SmEA and anti-IgE, induced discharge of histamine, IL-4, and IL-13, whereas pools 1 and 2 did not have any effect. Note that the production of IL-13 was studied in the absence of IL-3 because the latter cytokine alone induces considerable production of IL-13.

Together with the complete abrogation of SmEA-induced IL-4 induction through murine anti-His-IPSE antiserum (see

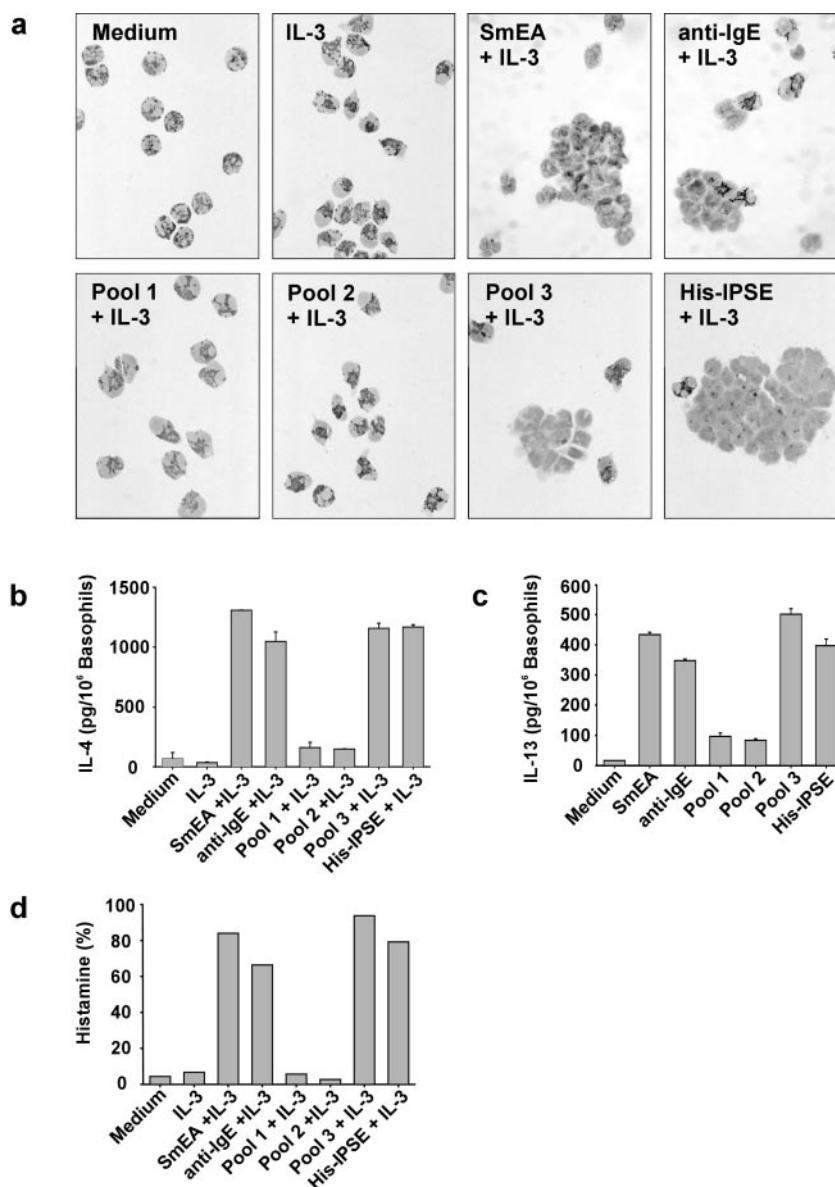


FIG. 6. Basophil degranulation and mediator release induced by IPSE. *a*, cytospin preparations of human basophils incubated for 30 min with medium (*Medium*), IL-3 (*IL-3*), or IL-3 + pools 1 (*Pool 1*), 2 (*Pool 2*), and 3 (*Pool 3*) (each 0.4 μ l/ml) from cation exchange chromatography of SmEA (fractions 6–10, 48–55, and 56–63, respectively) or with 24 ng/ml recombinant IPSE (*His-IPSE*). Pool 3 and His-IPSE (like SmEA and anti-IgE) cause degranulation and, as shown here, often agglutination, whereas IL-3 alone leads only to shape change. *b*, release of IL-4 from basophils following 4-h stimulation with medium, IL-3 (10 ng/ml), SmEA (10 μ g/ml) + IL-3, anti-IgE (100 ng/ml) + IL-3, pool 1, 2, or 3 (each 0.4 μ l/ml) + IL-3, or His-IPSE (24 ng/ml) + IL-3. *c*, IL-13 secretion by basophils after culture (40 h) with medium, SmEA, anti-IgE, pool 1, 2, or 3, or recombinant His-IPSE (all without IL-3; concentrations as for *b*). *d*, release of histamine (%) from basophils (conditions as for *b*). Representative experiments are shown.

above), these findings strongly indicate that IPSE is the only bioactive component in SmEA that activates basophils for degranulation, mediator release, and expression of immunoregulatory cytokines.

IPSE Is an IgE-binding Factor—Earlier studies had demonstrated that IgE has to be present on basophils for IPSE to become effective. To elucidate whether IPSE might act as an IgE-binding factor, blotting experiments were performed, where His-IPSE was submitted to SDS-PAGE, transferred to nitrocellulose membrane, and incubated with various concentrations of human IgE. Subsequent incubation with AP-conjugated anti-human-IgE antibody revealed that IgE was dose-dependently bound by IPSE. Analogous results were obtained when solid-phase IgE was incubated with increasing amounts of His-IPSE (Fig. 7). Biacore experiments confirmed these findings.³ Thus, these results clearly indicate that IPSE is an IgE-binding factor.

IPSE Is Enriched in and Secreted from the Subshell Area of the Schistosome Egg—We had earlier observed that IL-4-inducing activity is released from live schistosome eggs kept overnight at 37 °C in culture medium (11), suggesting that IPSE is

a secreted molecule. To further address this point, immunohistological localization of IPSE was performed in formalin-fixed liver sections of schistosome-infected mice using the monoclonal anti-His-IPSE antibody 74 1G2. This antibody stains the subshell area of schistosome eggs and binds to a variety of circumoval inflammatory cells both intracellularly and/or extracellularly. Interestingly, the subshell area stained strongest in eggs that were surrounded by relatively few inflammatory cells (developing granulomas?), with the cells themselves being not or minimally IPSE-positive. In contrast, the eggs stained weaker in large advanced granulomas, with many circumoval cells being strongly positive. This suggests that during granuloma development IPSE is continuously taken up and accumulated by circumoval cells. Analogous results (not shown) were obtained with a polyclonal rabbit antiserum to natural IPSE. On the other hand, the antibodies did not nonspecifically bind to liver cells or structures outside the egg granulomas. Moreover, there was no nonspecific binding of normal murine IgG (not shown) and/or the second antibody, respectively, to schistosome eggs or inflammatory cells (Fig. 8). In line with these findings, Ashton *et al.* (25) recently demonstrated that an antiserum raised against excretory/secretory components from schistosome eggs primarily stained the subshell area. Taken

³ S. Blindow, T. Weimar, and G. Schramm, unpublished data.

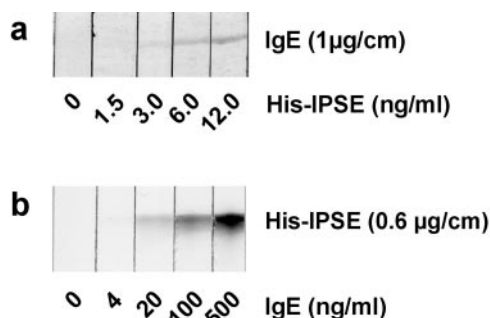


FIG. 7. Blot analysis showing the binding of IPSE to IgE. *a*, highly purified polyclonal human IgE (Biermann, Bad Nauheim, Germany) was submitted to SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with various concentrations of His-IPSE. Bound His-IPSE was detected with monoclonal anti-IPSE antibodies (clone 74 2G4; supernatant, diluted 1:50), and the latter was visualized by AP-conjugated goat anti-mouse IgG (1:10,000; Dianova) followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. *b*, His-IPSE was submitted to SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with various concentrations of IgE. Bound IgE was detected with AP-conjugated mouse monoclonal anti-human IgE (diluted 1:10,000; BD Pharmingen, Heidelberg, Germany) followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

together, we conclude that IPSE is enriched in the subshell area of the schistosome egg from which it is secreted into the surrounding tissue, thereby interacting with a variety of circumoval inflammatory cells possibly being internalized by some of them.

DISCUSSION

Here we describe the purification, N-terminal sequence analysis, cloning, and recombinant expression of IPSE, a glycoprotein secreted from *S. mansoni* eggs. IPSE triggers basophils from naive human donors to rapidly degranulate, release mediators, and express IL-4 and IL-13, the key cytokines controlling a Th2-type immune response and IgE synthesis.

The protocol for purifying IPSE was a combination of cation exchange chromatography and affinity chromatography with the lectin AAA. The purified material had a high specific activity and consisted of two bands of about 40 kDa as assessed by nonreducing SDS-PAGE. The purity of the natural bioactive material was further demonstrated by microsequencing a preparation containing the two bands, which revealed one unequivocal N-terminal aa sequence, suggesting that both represent posttranslational variants of the same protein. This conclusion was supported by the finding that all clones ($n = 12$) obtained from the two libraries were 100% identical.

For probing the SmE-libraries, a nondegenerate 30-mer oligonucleotide was used, corresponding to a region of 10 aa that was identical between the N-terminal sequence of purified IPSE and the homologous *S. mansoni* EST retrieved from the data base. Thus, if present, the cDNA corresponding to the EST should also have been recovered. However, the fact that from two different SmE-libraries 12 identical clones corresponding to IPSE were isolated suggests that the respective sequence represents a dominant form of this molecule, whereas another highly homologous *S. mansoni* gene either does not exist or is expressed in the egg stage only at a very low level.

The mature protein consists of 114 aa, without relevant sequence homologies with known proteins. Secondary structure predictions (PHD/PROF on the World Wide Web at maple.bioc.columbia.edu/predictprotein) indicate that the IPSE monomer forms a compact globular structure composed mainly of β -sheets, including a crystallin β/γ "Greek key" motif. Thus, IPSE may have some structural similarity with the γ -crystallin-like superfamily, and indeed an assignment to this

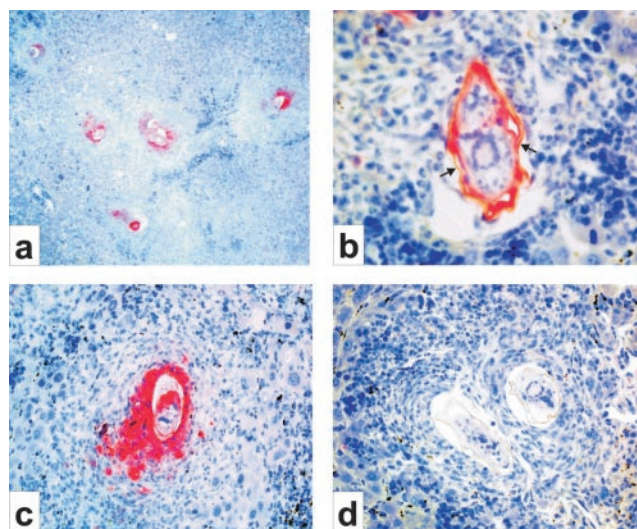


FIG. 8. Immunohistological detection of IPSE in *S. mansoni*-infected mouse liver. Liver sections of mice infected with *S. mansoni* were incubated with the murine monoclonal anti-His-IPSE antibody 74 1G2 (*a-c*) or with PBS (*d*). Bound antibody was detected with AP-conjugated F(ab)₂ goat anti-murine IgG followed by Neufuchsin and hematoxylin staining. *a*, general view showing liver tissue containing several schistosome egg granulomas. Note staining of eggs and/or circumoval inflammatory cells. *b*, longitudinal section of a schistosome egg in a small (developing?) granuloma with nearly exclusive staining of the subshell area; the arrows depict egg shell. *c*, longitudinal section of a schistosome egg in an advanced granuloma with extensive staining of circumoval cells and moderate staining of the egg. *d*, longitudinal and traverse section of two schistosome eggs; negative control.

superfamily was also obtained (E value: 4.4×10^{-5}) by using the Superfamily HMM Library and Genome Assignments server (available on the World Wide Web at supfam.org/SUPERFAMILY). As assessed by SDS-PAGE analysis performed under reducing and nonreducing conditions, recombinant IPSE spontaneously formed disulfide-linked homodimers, consistent with the presence of an uneven number of seven cysteines. Correspondingly, when natural IPSE was subjected to SDS-PAGE under reducing conditions, its apparent molecular mass decreased from 40 to 20 kDa. Given a calculated molecular mass of 26.4 kDa for recombinant homodimeric IPSE, natural IPSE appears to be a homodimeric glycoprotein with a glycan content of about 30%.

Clear evidence that the recombinant protein corresponds to IPSE came from functional studies. Like natural IPSE, the recombinant analogue triggered degranulation of basophils and caused release of histamine, IL-4, and IL-13. The high specific activity of His-IPSE (maximum at 0.75 nM) was in the same range as that of the natural protein. Moreover, the fusion protein formed homodimers just like natural IPSE. Cell activation experiments with whole SmEA, pooled SmEA fractions, and recombinant IPSE, respectively, strongly suggested that basophil activation by SmEA was due to IPSE and not to other components of SmEA. In line with these findings, agglutination of basophils correlated with the presence of IPSE and not the other components. Moreover, antibodies raised against His-IPSE were reactive with natural IPSE (Western blot) and functionally neutralized as well as immunodepleted natural IPSE from SmEA.

Although IPSE seemed to be a completely novel protein, the similarity of its appearance in cation exchange fractions and its apparent molecular weights under reducing and nonreducing conditions as well as its glycosylation suggested that IPSE might be identical with antigen α_1 found in *S. mansoni* eggs (26). This glycoprotein is a candidate antigen for serodiagnosis of *S. mansoni* infection but had thus far not been further

characterized. Indeed, Western blotting of antigen α_1 and IPSE, respectively, with anti- α_1 and anti-IPSE antibodies as well as N-terminal sequencing of antigen α_1 clearly indicates both molecules to be identical.⁴

It has been proposed that carbohydrate components of SmEA down-regulate Th1 responses (27) and are also required for SmEA-stimulated Th2 induction. The latter was demonstrated in a murine model of intranasal sensitization with deglycosylated (periodate-treated) SmEA that, compared with the native material, failed to induce IL-4, IL-5, IL-10, and SmEA-specific IgE production (28). Moreover, lacto-*N*-fucopentaose III, a predominant glycan component of SmEA that contains Lewis^x (Le^x), has been found to function as a Th2-inducing adjuvant in mice when covalently linked to human serum albumin (29). Since natural IPSE binds to AAA, a lectin specific for α 1-6-linked fucose (30) and for Fuc α 1-3-GlcNAc > Le^x > sialyl Le^x (31), it is possible that natural IPSE contains lacto-*N*-fucopentaose III. However, for the functional activity of IPSE being investigated here, carbohydrates are not necessary, since unglycosylated recombinant IPSE has a powerful IL-4-inducing effect. Moreover, lacto-*N*-fucopentaose III coupled to human serum albumin (Biocarb Chemicals, Lund, Sweden) did not activate human basophils when added over a wide concentration range (0.03–30 μ g/ml using 0.3 log increments; data not shown). Of course, our findings do not exclude the possibility that carbohydrates can contribute to a Th2 bias by mechanisms involving cell types other than basophils.

Regarding the mechanism by which IPSE activates basophils, earlier IgE stripping and resensitization experiments had revealed that IgE has to be present on the cells for IPSE to exert its effect (10, 11), suggesting that this schistosome-derived molecule acts via cross-linking of Fc ϵ RI-bound IgE. This assumption is supported by our blotting and Biacore experiments demonstrating dose-dependent binding of His-IPSE to solid-phase IgE and *vice versa*. Taken together, IPSE is an IgE-binding factor that activates basophils presumably via cross-linking receptor-bound IgE.

The type of interaction between IPSE and IgE is not yet known. Basically, it could be an antigen-specific, lectin-like, or B cell superantigen-like interaction. Antigen-specific binding is highly unlikely, since the basophils were from nonsensitized donors, and, in the case of the recombinant molecule, potentially cross-reactive carbohydrates were not involved. The possibility that IPSE acts as a lectin cannot be excluded, but its sequence does not show any similarity with known animal or plant lectins. Furthermore, although several lectins can activate basophils (32), the concentrations required are in the μ g/ml range. In contrast, the possibility that IPSE is a B cell superantigen requires consideration; B cell superantigens, such as HIV-1 gp120, protein Fv, and protein L (33, 34),² can bind to IgE and, as multivalent molecules, can activate basophils, resulting in the release of IL-4 and IL-13. Furthermore, release of IPSE-induced IL-4 can be inhibited in the presence of increasing concentrations of human polyclonal IgG. Polyclonal IgG contains various VH segment family members, and some of them may compete with corresponding VH segments of IgE for binding to IPSE. Experiments addressing these points are currently under way.

The *in vivo* effects of IPSE are not known yet. The pronounced IL-4 and IL-13 production triggered by this factor *in vitro* strongly suggests that IPSE might be involved in initiating and/or amplifying a Th2 response. Basophils, while accounting for only 1% of human peripheral blood leukocytes (35), have been found to produce more IL-4 during the first

6 h after antigen activation than lymphocytes (36) and are considered to be an initial source of IL-4 (4, 16, 37–39). Basophils are mobile blood cells and can readily reach sites of interaction with pathogens; they may thus be attracted to sites of schistosome egg deposition. Morphologically intact granulated basophils are rarely observed in schistosome egg granulomas, but in view of the powerful activating effect of IPSE, it is quite likely that basophils in this environment are degranulated (“ghost cells”) and thus are not easily recognized by conventional histochemical staining. Moreover, basophils share several chemokine receptors with eosinophils such as CCR2 and CCR3 (40–42), which are bound by the chemokines MCP-1, MCP-2, MCP-3, and MCP-4 and the chemokines MCP-3, MCP-4, RANTES, eotaxin I, and eotaxin II, respectively, suggesting that they could be important participants in the circumoval immune response, which is dominated by eosinophils for several weeks. Since IPSE is secreted by schistosome eggs, as concluded both from its presence in egg culture supernatants and from the immunohistological finding of IPSE on and in circumoval inflammatory cells, this factor will obviously interact also with basophils attracted to the schistosome egg granuloma.

In conclusion, IPSE is a parasitic glycoprotein with a novel and potent activating effect on human basophils. Because it can rapidly trigger basophils from nonsensitized human donors to release considerable amounts of IL-4, IPSE is a good molecular candidate for biasing the immune response toward the Th2 phenotype in the course of schistosome infection.

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⁴ G. Schramm and M. J. Doenhoff, unpublished data.

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