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(54) Title: PARASITE IMMUNOGLOBULIN BINDING PROTEINS, AND THEIR USE AS VACCINES

(57) Abstract

The present invention provides a protective metazoan parasite antigen, characterised in that said antigen is a protein capable of binding to host immunoglobulin, or a functionally equivalent variant, or antigenic fragment of precursor thereof, its preparation from metazoan parasites, its use in vaccine compositions, DNA sequences encoding it and its production by recombinant means.
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PARASITE IMMUNOGLOBULIN BINDING PROTEINS, AND THEIR USE AS VACCINES

The present invention relates to novel parasite antigens and their use in the control of diseases and injury caused by parasites in animals, particularly helminths and arthropod parasites, and especially ectoparasites in domestic animals.

Metazoan parasites, particularly arthropods and helminths, infect or infest a wide range of animals, including man, and are a widespread and significant source of disease and other injurious effects. Such parasites thus represent a considerable world-wide drain on economic resources. This is particularly true in animal husbandry, where parasitic infections of grazing animals, such as sheep and cattle, are often difficult and expensive to control, and may result in significant economic losses.

Particular mention may be made in this regard of many arthropod species, especially members of the arachnid and insect classes, and in particular ectoparasites such as the blood-feeding (haematophagous) insects, mites and ticks.

In addition to the direct parasitic effects, many blood-feeding arthropods are important vectors of a variety of pathogens, e.g. protozoa and viruses, of medical and veterinary significance. Livestock producers annually experience substantial losses due to haematophagous arthropods and the diseases they transmit.

Also worthy of particular mention are many helminth species, particularly nematodes, and especially those parasitising the gastro-intestinal tract of mammals. Thus for example blood feeding nematodes of the genus Haemonchus, parasites of ruminants, most notably sheep and cattle, are of considerable economic importance, as
are the non-blood feeding nematodes of the genera
Ostertagia and Teladorsagia.

Other parasitic helminths of economic importance
include the various species of the following helminth
genera: Trichostrongylus, Nematodirus, Dictyocaulus,
Cooperia, Ascaris, Dirofilaria, Trichuris, Strongylus,
Fasciola, Oesophagostomum, Bunostomum and
Metastrongylus. In addition to domestic livestock, pets
and humans may also be infected, not infrequently with
fatal results.

Control of helminth and arthropod parasites of
grazing livestock currently relies primarily on the use
of anthelmintics, endectosides, acaricides and/or
insecticides combined with pasture management. Such
techniques are often unsatisfactory firstly, because
these chemicals have to be administered frequently,
secondly because resistance against anthelmintic and
arthropodicidal chemicals is becoming increasingly
widespread and thirdly because appropriate pasture
management is often not possible on some farms and even
where it is, it can place constraints on the best use of
available grazing.

To overcome these problems, attempts have been made
to achieve immunological means of control. Although
there has been some success in identifying certain
protective antigens as potential vaccine candidates,
most notably in cattle ticks and the nematode
Haemonchus, this approach has proved difficult and,
other than for the cattle lungworm Dictyocaulus
viviparus, has yet to come to commercial fruition.

As far as arthropod parasites are concerned, the
literature contains few reports of proposed vaccines and
these concentrate mainly on cattle ticks, particularly
the tick Boophilus microplus. Thus, Johnson et al. in
1986 proposed that protective antigens against B.
microplus could be found in both soluble and insoluble
fractions of extracts derived from adult female ticks.
Since then reports have appeared in the literature proposing immunisation of cattle against ticks using
tick antigens derived from extracts of the gut of adult
ticks, tick larval extracts and from salivary gland
components (see for example Willadsen et al., 1989, Int.
J. Parasitol. 18(2): 183-189; Wong et al., 1990,
Parasite Immunology, 12: 75-83; Jackson et al., 1990,
Parasite Immunology, 12: 141-151).

As far as helminths are concerned, the most success
to date has been achieved with the protein doublet
H110D, an integral membrane protein isolated from the
gut of Haemonchus contortus and described by Munn in
WO88/00835. H110D now represents the most promising
anti-helminth vaccine candidate to date.

Other helminth integral gut membrane proteins are
also becoming recognised as potentially important
vaccine targets. Thus for example, contortin, a helical
polymeric extracellular protein associated with the
luminal surface of H. contortus intestinal cells, has
also been described and proposed as a vaccine, Munn et

A third Haemonchus gut membrane protein with
protective antigenic properties has also been discovered
and termed H45 (Munn and Smith, WO90/11086).

Whilst proteins such as those mentioned above can
be used as the basis for a vaccine against cattle ticks
or helminths such as Haemonchus, there is nonetheless a
continuing need for new and improved metazoan parasite
vaccines and in particular for a vaccine which may be
used across a broad range of helminth and/or arthropod
genera.

The present invention accordingly seeks to provide
novel antigens for use as metazoan parasite vaccines and
in particular as protective immunogens in the control of
diseases caused by arthropod and other metazoan
parasites.
More specifically, the present invention is based on the finding that arthropod parasites, and especially blood-feeding ectoparasites such as ticks, contain proteins capable of binding to host immunoglobulins. It is known that host proteins, including immunoglobulins, may be ingested by metazoan parasites during feeding, for example as part of a blood meal, and that such ingested immunoglobulin (Ig) may retain biological activity and be capable of binding to targets, for example internal organs within the parasite (see for example Ackerman et al., 1981, Journal of Parasitology 67, 737-740; Fujisaki et al., 1984, Annals of Tropical Medicine and Parasitology. 78, 449-550; Brossard & Rais, 1984, Experientia 40, 561-562; Ben-Yakir et al., 1986, in Morphology, Physiology and Behavioral Biology of Ticks (ed. Sauer, J.R. and Hair, J.A.), pp. 329-341. Chichester, UK: Ellis Horwood Ltd; Minoura et al., 1985, Experimental Parasitology 60, 355-363; Tracey-Patte et al., 1987, Research in Veterinary Science 43, 287-290; and Azad et al., 1989, Host Regulated Developmental Mechanisms in Vector Arthropods. pp163-170).

In the case of ticks, it has furthermore been observed that during feeding, the concentration of Ig in tick haemolymph increases (Ben-Yakir et al., 1987, supra). Following engorgement, Ig in the haemolymph disappears (Chinzei & Minoura, 1987, Medical and Veterinary Entomology 1, 409-416). However, the mechanism involved in the loss of Ig from the haemolymph has not been determined.

Studies leading to the present invention have now shown that metazoan parasites such as ticks are able to transport ingested host Ig from the gut, through the haemolymph and into their salivary glands, and excrete it in saliva, thereby removing it from the parasite body. This observation, coupled with the finding of immunoglobulin binding proteins (IGBPs) within the haemolymph and salivary glands of the parasite, suggests
that ticks and similar ectoparasites have evolved a mechanism for eliminating foreign proteins via the salivary gland, as a means of self-defence against the immune response of the host, and that this mechanism involves IGBPs.

Helminth and arthropod parasites frequently exhibit many similarities, for example in feeding behaviour and such like, and in many cases have shown themselves to be susceptible to similar types of vaccine, for example candidate vaccines using integral gut membrane proteins as so-called "hidden antigens" have been shown to be effective against both nematodes and ticks. Furthermore, both blood-feeding and non-blood feeding helminths have been shown to ingest host immunoglobulin. It is believed therefore, that helminth parasites may exhibit a similar defence mechanism, to evade the host's immune response.

The present invention is based on the concept of using such parasite immunoglobulin binding proteins capable of binding host Ig as a vaccine target, thereby depriving the parasite of an important means of defence against the immune response of the host.

According to one aspect, the present invention thus provides a protective metazoon parasite antigen, characterised in that said antigen is a protein capable of binding to host immunoglobulin, or a functionally equivalent variant, or antigenic fragment or precursor thereof.

A further aspect of the invention provides such protective antigens, and functionally-equivalent variants and antigenic fragments and precursors thereof, for use in stimulating an immune response against metazoon parasites in a human or non-human, preferably mammalian, especially preferably ruminant, animal.

A precursor for the antigen in question may take the form of a larger protein which is processed, e.g. by proteolysis to yield the antigen per se.
The novel antigens of the invention are not recognised by sera from naturally immune animals. In other words, they are not normally, in native form, accessible to the immune system of the infected host and are thus "hidden", "concealed" or "cryptic" antigens.

The term "protective antigens" or "protective antigenic activity" as used herein defines those antigens and their fragments, capable of generating a host-protective, i.e. immunogenic, immune response, that is a response by the host which leads to generation of immune effector molecules, antibodies or cells which damage, inhibit or kill the parasite and thereby "protect" the host from clinical or sub-clinical disease and loss of productivity. Such a protective immune response may commonly be manifested by the generation of antibodies which are able to inhibit the metabolic function of the parasite, leading to reduction of feeding performance and growth, inhibited reproduction and/or death.

In the case of certain metazoan parasites, notably the helminths, and those ectoparasites that are obligate feeders eg. lice, keds and mites or those that feed for some period of time on the host eg. blowfly, warble fly, screw worm, and bot larvae, horn/buffalo flies, fleas and ticks, the host protective immune response may result in death, debilitation and/or expulsion of the parasite from the infected animal. In other words the parasitic burden on the infected animal is reduced and the infected animal itself directly reaps the benefit of its own immune response. In other cases however, an "epidemiological" effect may be observed whereby the immediate host may not necessarily itself benefit significantly from its immune response. For example as a result of feeding on immunised hosts, populations of parasites in a given area may be reduced and/or attenuated, thereby protecting later herds/flocks of the host or other hosts. Maintaining the immunisation
approach through a number of seasons will continue to reduce the threat of parasite effects and disease. This is particularly true for arthropod ectoparasites such as ticks, flies, etc. which may feed and move from host to host. In this way inhibitory antibodies ingested by the parasite may exert their effect after the parasite has left that host, but by killing, debilitating or reducing the fecundity of the parasite, other members of the host animal group and other host species may be protected.

As mentioned above, included within the scope of the invention are functionally-equivalent variants of the novel antigens and their fragments and precursors. "Functionally-equivalent" is used herein to define proteins related to or derived from the native protein, where the amino acid sequence has been modified by single or multiple amino acid substitution, addition and/or deletion and also sequences where the amino acids have been chemically modified, including by deglycosylation or glycosylation, but which nonetheless retain protective antigenic activity eg. are capable of raising host protective antibodies and/or functional immunity against the parasites. Within the meaning of "addition" variants are included amino and/or carboxy terminal fusion proteins or polypeptides, comprising an additional protein or polypeptide fused to the IGBP antigen sequence. Such functionally-equivalent variants mentioned above include natural biological variations (eg. allelic variants or geographical variations within a species) and derivatives prepared using known techniques. For example, functionally-equivalent proteins may be prepared either by chemical peptide synthesis or in recombinant form using the known techniques of site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids. Functionally-equivalent variants according to the invention also include analogues in different parasite genera or species.
Certain IGBP antigens of the present invention are believed to be associated with the membranes of metazoan parasites in their native form, and in particular, to be integral membrane proteins. Detergent extraction studies have shown that strong detergents such as Triton X-100° are required to solubilise and extract such antigens. Other IGBP antigens according to the invention may exhibit characteristics typical of secreted proteins. Thus different families of IGBP antigens may exist, having different solubility characteristics.

In the case of arthropod parasites such as ticks, the IGBP antigens of the invention have, as mentioned above, been shown to be located in the haemolymph and salivary glands. Such antigens have been shown to be present both in male and female ticks and in a variety of tick species.

As will be described in more detail below, different IGBPs may be expressed in different parts or organs of the parasite, and at different stages, for example stages of feeding.

In the case of the tick *Rhipicephalus appendiculatus*, SDS-PAGE and immunoblotting studies have shown that a range of IGBP antigens of different molecular weight may be expressed in the haemolymph and salivary glands, and that the pattern of expression in the different organs may vary with time from commencement of feeding, with further proteins becoming expressed as feeding progresses, and the expression of other IGBPs diminishing. Furthermore, the pattern of expression may vary between male and female ticks. Further details are described in the Examples below.

IGBPs of varying molecular weights and stages of expression have also been shown in other ixodid ticks, for example *Amblyomma variegatum* and *Ixodes hexagonus*. A summary of molecular weights and location of the various proteins is provided in Table 1 in Example 4.
In the tick *Rhipicephalus appendiculatus*, particular mention may be made of three dominant proteins, herein termed MA, MB and MC of molecular weight about 29kD, 25kD and 21kD respectively which appear in the salivary gland extracts of feeding males. cDNA clones encoding these proteins have been prepared and sequenced and the nucleotide sequences and their translations are shown in Figures 27A to 27C. Partial N-terminal amino acid sequences are also shown in Figures 20, 28 and 29.

The metazoan parasites of particular concern in the present invention include especially helminths and arthropod parasites, in the case of the latter, particularly ectoparasites and especially, but not exclusively, the blood-feeding insect and acarine parasites, e.g. biting flies, cattle ticks and mites. As representative of such arthropod parasites may be mentioned for example, ticks of the species *Boophilus*, *Amblyomma*, *Argas*, *Rhipicephalus*, *Hyalomma*, *Ornithodorus*, *Dermacentor*, *Ixodes*; flies, particularly the myiasis, sucking and biting flies, such as *Oestrus ovis*, *Gasterophilus* spp, *Chrysomyia* spp, *Calliphora* spp, *Hypoderma* spp, *Dermatobia* spp, *Cochliomyia* spp, *Stomoxys calcitrans*, *Hydrotaea irritans*, *Simulium* spp, *Lyperosia irritans*, *Haematobia* spp, *Tabanus* spp, *Phlebotomus* spp and *Glossina* spp, lice eg. *Haematopinus eurysternus*, *Linognathus vituli*, *Solenopotes capillatus*, *Linognathus ovillus*, and *Menacanthus* spp; mites such as *Psoroptes* spp, *Demodex* spp, *Sarcoptes* spp, *Chorioptes* spp, *Psorergates* spp, *Dermanyssus* spp, *Ornithonyssus* spp, *Otodectes* spp and *Notoedres* spp; fleas eg. *Ctenocephalides canis* and *C. felis*; keds eg. *Melophagus ovinus* and bugs such as *Cimex* spp.

Helminth parasites may be any of those known to infect or infest man and his domestic animals. Representative helminth parasites include those mentioned above. Preferably, however the helminths will be nematodes, especially preferably gastro-intestinal
nematodes, including for example Haemonchus, Ostertagia and Trichostrongylus. (For the avoidance of doubt the term "Ostertagia" as used herein includes Teladorsagia sp.)

The antigens according to the invention may be prepared by extraction from parasites using conventional biochemical and surgical techniques such as homogenisation, either of the whole or part of the parasite, followed by isolation of the desired antigen by conventional purification techniques such as centrifugation, selective precipitation, chromatography and the like. Thus for example the antigens may be extracted from organs such as the salivary glands or from body fluids such as saliva or haemolymph. A preferred process takes advantage of the fact that the target molecules have particular selective binding activities by using an affinity chromatography system in which specific ligands are immobilised on a solid phase matrix.

Thus the invention also provides a process for the preparation of a vaccine for use in immunising a human or non-human, preferably mammalian, animal against a metazoan parasite infection, said process comprising preparing an extract of said parasite containing at least one protective antigen as defined above, purifying said antigen therefrom by binding said antigen to an immobilized phase including a specific binding partner for the antigen and subsequently eluting said antigen from said immobilized phase. Suitable specific binding partners include in particular host immunoglobulins.

The parasite extract which is subjected to affinity chromatography as described above may for example be a detergent extract, or more particularly an integral membrane fraction.

Such an integral membrane fraction of the parasite can readily be prepared using techniques well known in the art eg. detergent extraction for example with Triton
X-100. Affinity chromatography techniques are likewise well known in the art, and include batch and column-based systems. A range of solid phases to which the binding partner may be attached, may be used eg. gels such as agarose or sepharose.

Viewed from a different aspect, the invention can also be seen to provide use of a metazoan parasite antigen as hereinbefore defined, and fragments, precursors and functionally-equivalent variants thereof, for the preparation of a vaccine composition for use in stimulating an immune response against metazoan parasites in a human or non-human, animal.

The invention also provides a vaccine composition for stimulating an immune response against metazoan parasites in a human or non-human animal comprising one or more antigens, antigenic fragments, precursors or functionally-equivalent variants thereof, as defined above, together with a pharmaceutically acceptable carrier or diluent, and a method of stimulating an immune response against metazoan parasites in a human or non-human animal, comprising administering to said animal a vaccine composition as defined above.

The animal preferably is mammalian and more preferably a ruminant.

As mentioned above, antigens according to the invention may be obtained from a range of metazoan parasite genera. Such antigens may be used to prepare vaccines against a range of metazoan parasites including any of those mentioned above. Preferred are those antigens, so called "broad spectrum" antigens, which are capable of stimulating host protective immune responses against, in addition to the parasite from which they were isolated, a broad range of other parasites.

As mentioned above, one of the ways in which the antigens of the invention may exert their host protective effects is by raising inhibitory antibodies which inhibit the growth, maintenance and/or development
of the parasite. Such antibodies and their antigen-binding fragments (e.g. F(\text{ab})\text{\textregistered}_2, Fab and Fv fragments i.e. fragments of the "variable" region of the antibody, which comprises the antigen binding site) which may be mono- or polyclonal, form a further aspect of the invention, as do vaccine compositions containing them and their use in the preparation of vaccine compositions for use in immunising hosts against parasites. Such inhibitory antibodies may be raised by use of idiotypic antibodies. Anti-idiotypic antibodies may be used as immunogens in vaccines.

Accordingly, a further aspect of the invention provides an antibody, or antigen-binding fragment thereof, which is capable of selectively binding to an antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as hereinbefore defined, or to the idiootype of a said antigen-binding antibody.

In addition to the extraction and isolation techniques mentioned above, the antigens may be prepared by recombinant DNA technology using standard techniques, such as those described for example by Sambrook et al., 1989, (Molecular Cloning, a laboratory manual 2nd Edition, Cold Spring Harbor Press).

Nucleic acid molecules comprising a nucleotide sequence encoding the antigens of the invention thus form further aspects of the invention.

As mentioned above, sequence studies have been performed of cDNA clones corresponding to the MA, MB and MC IGBP\textsuperscript{s} of male \textit{R. appendiculatus}. The sequences of these clones are shown in Figures 27A to 27C.

Accordingly, a preferred nucleic acid molecule according to the invention comprises one or more nucleotide sequences substantially corresponding to all or a portion of the nucleotide sequence shown in any one of Figures 27A to 27C or a sequence which is degenerate or substantially homologous therewith or which hybridises with said sequence.
Nucleic acid molecules according to the invention may be single or double stranded DNA, cDNA or RNA, preferably DNA, and include degenerate, substantially homologous and hybridising sequences which are capable of coding for the antigen or antigen fragment or precursor concerned. By "substantially homologous" is meant sequences displaying at least 60%, preferably at least 70% or 80% sequence homology. Hybridising sequences included within the scope of the invention are those binding under non-stringent conditions (6 x SSC/50% formamide at room temperature) and washed under conditions of low stringency (2 x SSC, room temperature, more preferably 2 x SCC, 42°C) or conditions of higher stringency eg. 2 x SSC, 65°C (where SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2), as well as those which, but for the degeneracy of the code, would hybridise under the above-mentioned conditions.

Derivatives of nucleotide sequences capable of encoding antigenically active antigens or antigen variants according to the invention may be obtained by using conventional methods well known in the art.

Antigens according to the invention may be prepared in recombinant form by expression in a host cell containing a recombinant DNA molecule which comprises a nucleotide sequence as broadly defined above, operatively linked to an expression control sequence, or a recombinant DNA cloning vehicle or vector containing such a recombinant DNA molecule. Synthetic polypeptides expressed in this manner form a further aspect of this invention (the term "polypeptide" is used herein to include both full-length protein and shorter length peptide sequences).

The antigen so expressed may be a fusion polypeptide comprising all or a portion of an antigen according to the invention and an additional polypeptide coded for by the DNA of the recombinant molecule thereto. This may for example be β-galactosidase,
glutathione-S-transferase, hepatitis core antigen or any of the other polypeptides commonly employed in fusion proteins in the art. Such fusion proteins may also comprise the antigen in a form which may be secreted i.e. the antigen may be expressed together with signal and secretion-directing sequences.

Other aspects of the invention thus include cloning and expression vectors containing the DNA coding for an antigen of the invention and methods for preparing recombinant nucleic acid molecules according to the invention, comprising inserting nucleotide sequences encoding the antigen into vector nucleic acid, eg. vector DNA. Such expression vectors include appropriate control sequences such as for example translational (eg. start and stop codons, ribosomal binding sites) and transcriptional control elements (eg. promoter-operator regions, termination stop sequences) linked in matching reading frame with the nucleic acid molecules of the invention. Optional further components of such vectors include secretion signalling and processing sequences.

Vectors according to the invention may include plasmids and viruses (including both bacteriophage and eukaryotic viruses) according to techniques well known and documented in the art, and may be expressed in a variety of different expression systems, also well known and documented in the art. Suitable viral vectors include baculovirus and also adenovirus and vaccinia viruses. Many other viral vectors are described in the art.

A variety of techniques are known and may be used to introduce such vectors into prokaryotic or eukaryotic cells for expression, or into germ line or somatic cells to form transgenic animals. Suitable transformation or transfection techniques are well described in the literature.

The invention also includes transformed or transfected prokaryotic or eukaryotic host cells, or
transgenic organisms containing a nucleic acid molecule according to the invention as defined above. Such host cells may for example include prokaryotic cells such as E. coli, eukaryotic cells such as yeasts or the baculovirus-insect cell system, transformed mammalian cells and transgenic animals and plants. Particular mention may be made of transgenic nematodes (see for example Fire, 1986, EMBO J., 5. 2673 for a discussion of a transgenic system for the nematode Caenorhabditis).

A further aspect of the invention provides a method for preparing an antigen of the invention as hereinbefore defined, which comprises culturing a host cell containing a nucleic acid molecule encoding all or a portion of said antigen, under conditions whereby said antigen is expressed and recovering said antigen thus produced.

The antigens of the invention and functionally equivalent antigen variants may also be prepared by chemical means, such as the well known Merrifield solid phase synthesis procedure.

Water soluble derivatives of the novel antigens discussed above form a further aspect of the invention. Such soluble forms may be obtained by proteolytic digestion.

A vaccine composition may be prepared according to the invention by methods well known in the art of vaccine manufacture. Traditional vaccine formulations may comprise one or more antigens or antibodies according to the invention together, where appropriate, with one or more suitable adjuvants eg. aluminium hydroxide, saponin, quill A, or more purified forms thereof, muramyl dipeptide, mineral or vegetable oils, Novasomes or non-ionic block co-polymers or DEAE dextran, in the presence of one or more pharmaceutically acceptable carriers or diluents. Suitable carriers include liquid media such as saline solution appropriate for use as vehicles to introduce the peptides or
polypeptides into an animal or patient. Additional components such as preservatives may be included.

An alternative vaccine formulation may comprise a virus or host cell eg. a microorganism (eg. vaccinia virus, adenovirus or Salmonella) which may be live, killed or attenuated, having inserted therein a nucleic acid molecule (eg. a DNA molecule) according to this invention for stimulation of an immune response directed against polypeptides encoded by the inserted nucleic acid molecule.

Administration of the vaccine composition may take place by any of the conventional routes, eg. orally or parenterally such as by intramuscular injection, optionally at intervals eg. two injections at a 7-35 day interval.

The antigens may be used according to the invention in combination with other protective antigens obtained from the same or different parasite species. Such a combined vaccine composition may contain smaller amounts of the various antigens than an individual vaccine preparation, containing just the antigen in question. Combined vaccines are beneficial where there is a likelihood that "adaptive selection" of the parasite may occur when a single antigen vaccine is used.

Animals which may benefit from the present invention may be any human or non-human animal, but companion animals, particularly dogs and cats and domestic animals, especially ruminants are preferred. Particular mention may be made of sheep, cattle, pigs, deer and goats.

The invention will now be described in more detail with particular reference to antigens from ticks, and especially the tick *R. appendiculatus*. While not limiting the generality of the invention as defined above, it will be understood that IGBP antigens from blood feeding ectoparasites, and especially ticks, represent a preferred aspect of the invention. The
ixodid tick, *R. appendiculatus* is of particular importance since it is a vector of East Coast Fever, one of the most important livestock diseases in Africa.

In the following non-limiting Examples, the Figures represent:

**FIGURE 1** shows total protein concentration in the salivary gland extracts of ticks at different days of feeding. Concentrations were determined from pools of extracts derived from 30 ticks collected at 2 day intervals of feeding, and 110 unfed ticks;

**FIGURE 2** shows a silver stained gel of total proteins in salivary gland extracts and saliva. Salivary gland extracts were derived from unfed ticks (SGO), and ticks collected at 2 day intervals of feeding (SG2 to SG8); saliva was collected on days 6 and 8 of feeding (S6, S8). M, molecular weight markers (Sigma): bovine serum albumin (66 Kd), ovalbumin (45 Kd), glyceraldehyde-3-phosphate dehydrogenase (36 Kd), carbonic anhydrase (29 Kd), trypsinogen, 24 Kd, soybean trypsin inhibitor (20.1 Kd), α-lactalbumin (14.2 Kd);

**FIGURE 3** shows a silver stained gel of total proteins in haemolymph collected from unfed ticks (HO), and at 2 day intervals of feeding (H2 to H8). 10% SDS-polyacrylamide gel with reducing conditions;

**FIGURE 4** shows an immunoblot of SGED6 using control guinea pig serum;

**FIGURE 5** shows an immunoblot using tick immune serum with saliva of day 6 (S6), and salivary gland extracts (SG) and haemolymph (H) of unfed and 6 day feeding ticks;

**FIGURE 6** shows an immunoblot using SGEDO antiserum with salivary gland extracts (SG) and saliva (S) of unfed and feeding ticks;

**FIGURE 7** shows an immunoblot using SGEDO antiserum with haemolymph (H) of unfed and feeding ticks;

**FIGURE 8** shows an immunoblot using SGED6 antiserum with salivary gland extracts (SG) and saliva (S) of
unfed and feeding ticks;

FIGURE 9 shows an immunoblot using SGED6 antiserum with haemolymph (H) of unfed and feeding ticks;

FIGURE 10 shows an immunoblot using anti-guinea pig IgG conjugate with SGED0 to SGED8 or guinea pig serum. A, guinea pig serum (2 μg of protein); B, guinea pig serum (1 μg of protein); C, molecular weight markers; D, SGED0; E, SGED2; F, SGED4; G, SGED6; H, SGED8;

FIGURE 11 shows the relative concentrations of guinea pig IgG (ng protein/μg total protein of tick sample) in female tick haemolymph D0 to D8, SGE D0 to D8, and saliva D4 to D8. O, saliva; □, haemolymph; A, SGE;

FIGURE 12 shows the antibody activity of host IgG in haemolymph D6 and SGED6 of female tick. A, titre of anti-E. coli activity in diluted serum and tick samples; B, amount of total IgG in diluted anti-serum and tick samples;

FIGURE 13 shows a Coomassie blue stained polyacrylamide gel of fractions collected from a guinea pig IgG linked agarose column. A, column washings before elution with acid pH buffer; B, eluted haemolymph proteins (day 6 of feeding); C, eluted SGED6 proteins; D, molecular weight markers;

FIGURE 14 shows a salivary gland extract of unfed female R. appendiculatus examined by SDS-PAGE; Lane 1: high MW markers.
Lane 2 (last wash fraction of pH 7.0): the final 2 ml fraction of the TBS-Triton wash was collected and examined by SDS-PAGE to ensure that the column was washed clean before elution with GBS pH 2.6.
Lanes 3 & 4 (agarose-6B fractions 1 & 2): a 1 ml agarose-6B column was used to check the procedures with tick samples to make sure that tick proteins were not retained by the agarose column. Fractions 1 & 2 were the first and second fractions of the GBS pH 2.6 elution (1 ml each);
Lane 5 (agarose-IgG washed by pH 2.6): the agarose-IgG column was washed with GBS pH 2.6 before adding the tick sample. The bands show the IgG background due to some of the IgG that did not link tightly with the agarose. Lanes 6 & 7 (IGBP fractions 1 & 2): IGBPs in female unfed SGE eluted with GBS pH 2.6 (first and second 1 ml fractions).
Lane 8: low MW markers.
Lane 9: total proteins of female SGED0;

**FIGURE 15** shows the salivary gland extract of unfed male *R. appendiculatus* examined by SDS-PAGE.
Lane 1: low MW markers.
Lane 2: last fraction of TBS pH 7.0 wash.
Lanes 3 & 4: IGBP fractions 1 & 2 (see Fig. 14, lanes 6 & 7).
Lane 5: agarose-IgG washed with GBS pH 2.6 (see Fig. 14, lane 5).
Lane 6: low MW markers.
Lane 7: total proteins of male SGED0;

**FIGURE 16** shows a salivary gland extract of female *R. appendiculatus* fed for 6 days and examined by SDS-PAGE.
Lane 1: total proteins of female SGED6.
Lane 2: MW marker.
Lanes 3 & 4: IGBP in female SGED6, first and second 1 ml GBS pH 2.6 elutions.
Lane 5: last wash with TBS pH 7.0.
Lane 6: MW marker.
Lane 7: IgG-agarose washed with GBS pH 2.6 (background of the column);

**FIGURE 17** shows SDS-PAGE analysis of salivary glands of male *R. appendiculatus* after 6 days of feeding.
Lane 1: MW markers.
Lanes 2, 5, 8: last 1 ml of TBS pH 7.0 wash.
Lanes 3, 6, 9: IGBPs eluted with 1 ml GBS pH 2.6.
Lanes 4, 7, 10 (control): IgG-agarose column washed with
GBS pH 2.6 (background of the column).
Lane 11: MW markers.
Lane 12: total male SGED6 proteins.

This Figure also shows that the IGBPs in day 6 male
\textit{R. appendiculatus} also bind to IgG of other vertebrate
species.
Lanes 2,3,4: guinea pig IgG-agarose column.
Lanes 5,6,7: human IgG-agarose column.
Lanes 8,9,10: bovine IgG-agarose column.

\textbf{FIGURE 18} shows SDS-PAGE analysis of IGBPs in
salivary gland of other tick species.
Lane 1: \textit{Amblyomma variegatum} unfed female, total
proteins in SGED0.
Lane 2: MW markers.
Lane 3: \textit{Amblyomma variegatum} unfed female, IGBPs in
SGED0.
Lane 4: \textit{Amblyomma variegatum} unfed female, last wash
with TBS pH 7.0.
Lane 5: IgG-agarose washed with GBS pH 2.6 (background
of column).
Lane 6: \textit{Amblyomma variegatum} unfed male, IGBPs in SGED0.
Lane 7: \textit{Amblyomma variegatum} unfed male, last wash with
TBS pH 7.0.
Lane 8: MW markers.
Lane 9: \textit{Amblyomma variegatum} unfed male, total proteins
in SGED0.
Lane 10: \textit{Ixodes hexagonus} unfed male and female, IGBPs
in SGED0.
Lane 11: IgG-agarose washed with GBS pH 2.6 (background
of column).
Lane 12: MW markers.
Lane 13: \textit{Ixodes hexagonus} unfed male and female, total
proteins in SGED0;

\textbf{FIGURE 19} shows SDS-PAGE analysis of IGBP in
haemolymph of \textit{R. appendiculatus}.
Lane 1: MW markers.
Lane 2: Last wash with TBS pH 7.0>
Lane 3: IgG-agarose washed with GBS pH 2.6 (background of the column).
Lane 4: IGBP in haemolymph of unfed females.
Lane 5: IGBP in haemolymph of females fed for 6 days.
Lane 6: IGBP in haemolymph of males fed for 6 days.
Lane 7: MW markers.
Lane 8, 9, 10: total protein of haemolymph from female D0 and D6, and male D6, respectively.
Lane 11: MW markers.

**FIGURE 20** shows an alignment of the amino acid sequences determined for 21 kD and 25 kD male *R. appendiculatus* salivary gland IGBPs.

**FIGURE 21** shows a Western blot of IGBPs in SGE06 of male *R. appendiculatus* with antisera raised against IGBP.
Lane 1: MW markers.
Lane 2: antiserum of guinea pig 523 against IGBP MC.
Lane 3: antiserum of guinea pig 524 against IGBP MC.
Lane 4: antiserum of guinea pig 525 against IGBP MB and MA.
Lane 5: antiserum of guinea pig 526 against IGBP MA.

**FIGURE 22** shows SDS-PAGE analysis of IGBPs isolated from SGE of male *R. appendiculatus* ticks after 6 days of feeding.
Lane 1: Total protein profile of SGE day 6.
Lane 2: IGBP profile of eluted column (12.5% resolving gel).
Lane 3: molecular weight markers.

**FIGURE 23** shows immunoblotting of male day 6 SGE with anti-IGBP sera.
Lanes 1 and 8: pre-stained protein markers (Stratagene).
Lanes 2 to 4: immunoblots treated with sodium periodate.
Lanes 5 to 7: untreated immunoblots.
Lanes 2 and 5: GP 523 serum raised against IGBP-MC.
Lanes 3 and 6: GP 525 serum against IGBP-MB.
Lanes 4 and 7: GP 526 serum against IGBP-MA.
FIGURE 24 shows binding of IGBPs in day 6 male R. appendiculatus to either bovine, human or guinea pig IgG.
Lanes 1, 2, 3: bovine IgG-agarose column.
Lanes 4, 5, 6: human IgG-agarose column.
Lanes 7, 8, 9: guinea pig IgG-agarose column.
Lanes 1, 4, 7: untreated IgG-column washed with GBS (pH 2.6).
Lanes 2, 5, 8: IGBPs eluted with GBS (pH 2.6).
Lanes 3, 6, 9: Last wash of TBS-Triton (pH 7.0).
Lane 10: molecular weight markers.

FIGURE 25 shows protein profiles of male and female SGE collected on days 0 to 8 of the feeding period.
Lane 1: guinea pig IgG (only the heavy chain was detected).
Lanes 2 to 6: male SGE of day 8, 6, 4, 2, 0 of feeding, respectively.
Lanes 7 to 11: female SGE of day 8, 6, 4, 2, 0 of feeding, respectively.
Lane 12: molecular weight markers.

FIGURE 26 shows immunoblotting of male and female SGE with anti-IGBP sera.
Lane 1: guinea pig IgG.
Lanes 2 to 6: male SGE of day 8, 6, 4, 2, 0 of feeding, respectively.
Lanes 6 to 10: female SGE of day 8, 6, 4, 2, 0 of feeding, respectively.

FIGURE 27 shows the sequences of IGBPs:
(A): IGBP-MA;
(B): IGBP-MB;
(C): IGBP-MC;
(underlined residues indicate those which are identical in the N-terminal sequence and the translated sequence).

FIGURE 28 shows the signal sequences of IGBPs as predicted by SIGCLEAVE:
(A): IGBP-MA (Report scores over 3.50
Maximum score 1.5 at residue 8)
(B): IGBP-MB (Report scores over 3.50
Maximum score 6.5 at residue 16)
(C): IGBP-MB (Report scores over 3.50
Maximum score 9.0 at residue 21).

**FIGURE 29** shows BESTFIT of IGBP-MC + IGBP-MB,
wherein lines between bases indicate identical residues.

**FIGURE 30** shows protein profiles of SGE from male
R. appendiculatus ticks examined either immediately
after feeding was curtailed or 7 days later.
Lane 1: molecular weight markers.
Lanes 2, 3, 5, 7 and 9: proteins in the SGEs of unfed male ticks (day 0) and ticks fed for 2, 4, 6 or 8 days, respectively.
Lanes 4, 6, 8 and 10: proteins in the SGEs of male R. appendiculatus ticks examined 7 days after feeding for 2, 4, 6 or 8 days on guinea pigs.

**FIGURE 31** shows IGBP profiles of SGE from male ticks examined by immunoblotting either immediately after feeding was curtailed or 7 days later.
Lane 1: molecular weight markers.
Lanes 2, 3, 5, 7 and 9: IGBPs in the SGEs of unfed male R. appendiculatus ticks (day 0) and ticks fed for 2, 4, 6 or 8 days, respectively.
Lanes 4, 6, 8 and 10: IGBPs in the SGEs of male R. appendiculatus ticks examined 7 days after feeding for 2, 4, 6 or 8 days on guinea pigs.

**FIGURE 32** shows protein profiles of IGBPs examined either immediately after feeding was curtailed or 7 days later.
Lanes 1 and 2: IGBP-MA in SGE of ticks fed for 6 days (lane 1) and SGE of ticks fed for 6 days and then examined 7 days later (lane 2) developed by anti-IGBP-MA serum (guinea pig 526).
Lanes 3 and 4: IGBP-MB in SGE of ticks fed for 6 days (lane 3) and SGE of ticks fed for 6 days and then examined 7 days later (lane 4) developed by anti-IGBP-MB and MA serum (guinea pigs 525).
Lanes 5 and 6: IGBP-MC in SGE of ticks fed for 6 days (lane 5) and SGE of ticks fed for 6 days and then examined 7 days later (lane 6) developed by anti-IGBP-MC serum (guinea pig 523).
Lane 7: molecular weight markers.
EXAMPLE 1

Comparison of the Proteins in Salivary Glands, Saliva, and Haemolymph of Rhipicephalus appendiculatus Female Ticks During Feeding

Three preparations of guinea pig immune serum were used to compare the proteins in salivary glands, saliva and haemolymph of Rhipicephalus appendiculatus female ticks: (i) tick immune serum obtained from guinea pigs that had been repeatedly infested with R. appendiculatus nymphs, (ii) serum of guinea pigs immunised with soluble denatured salivary gland extracts (SGE) derived from unfed (day 0) R. appendiculatus female ticks, and (iii) serum of guinea pigs immunised with soluble denatured SGE derived from partially fed (day 6) female ticks. Immunoblotting with these sera following SDS-polyacrylamide gel electrophoresis was undertaken to examine the antigen profiles during the course of tick feeding on guinea pigs.

Preliminary studies using either antisera from guinea pigs inoculated with native salivary gland extract, or serum from tick immune guinea pigs, identified comparatively few proteins. In order to identify a greater number of proteins in SDS-polyacrylamide gel profiles as a prelude to isolating and characterising specific tick saliva proteins, guinea pigs were immunised with the soluble denatured SGE proteins derived from SDS-polyacrylamide gels. The resulting antisera were used to detect proteins in tick haemolymph, SGE, and saliva. The results enabled us to compare changes in protein content at different stages of tick feeding and to distinguish apparently common and unique proteins in haemolymph, salivary glands, and saliva.
MATERIALS AND METHODS

Ticks and Guinea Pigs

A laboratory colony of *R. appendiculatus* was maintained by feeding all three parasitic stages on the shaved backs of Dunkin Hartley guinea pigs (Tuck & Sons Ltd., Essex). Quiescent ticks were maintained in perforated tubes held in a desiccator at 28°C and 85% relative humidity (Jones et al., 1988, Journal of Animal Technology 39, 99-106). Equal numbers of male and female ticks were fed together for the experiments. Once they had fed for the desired period, females were removed from the guinea pigs and rinsed in water for at least three times to remove any superficial blood contamination.

Collection of Tick Haemolymph

A series of haemolymph samples of unfed and partially fed ticks was obtained. Haemolymph was collected into capillary tubes after cutting one or more legs (Ben-Yakir et al., 1986, supra) and immediately pooled and diluted into cold phosphate buffered saline (PBS: 10 mM phosphate, 140 mM NaCl, pH 7.3). Protein concentration was determined using the Bio-Rad Protein Assay Kit with bovine serum albumin as the standard. The diluted haemolymph was then denatured in SDS-PAGE sample buffer (5% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, and 0.0625 mM Tris-HCl, pH 6.8) by boiling for 2 minutes, and stored at -20°C before use.

Preparation of Soluble Salivary Gland Extracts

Salivary glands were dissected from either unfed or partially fed ticks in cold PBS, rinsed in fresh PBS, and then pooled and homogenized in PBS containing 1 mM
PMSF (phenylmethane-sulfonyl fluoride) at 4°C. The homogenate was centrifuged at 10,000g for 10 minutes at room temperature and the concentration of total proteins in the supernatant fluid determined. This soluble SGE was denatured by boiling for 2 minutes in SDS-PAGE sample buffer and stored at -20°C. Extracts prepared from salivary glands of unfed ticks, and from ticks collected at 2 day intervals of feeding, were labelled SGED0, SGED2, etc.

Collection of Tick Saliva

Salivation was induced by injecting female ticks parenterally with 25 µl/100 mg body weight of dopamine hydrochloride (2mM dopamine in PBS) within 1 hour of removal of the ticks from the guinea pig host. A capillary tube (10µl) was placed over the chelicerae and hypostome to collect the saliva (Kaufman, 1978, Am. J. Physiol. 235, R76-R81). Saliva was pooled and the protein concentration determined. The samples were then denatured (see above) and stored at -20°C.

Gel Electrophoresis

Normal SDS-PAGE (Harris & Angal, 1989, Protein purification methods. 1. Proteins Purification, pp. 21-27, IRL Press, Oxford) was used to separate tick proteins; 15µl of total protein were loaded in each lane. The gels were run at 100V in a 3% stacking gel and at 200V in a 12.5% resolving gel unless otherwise stated. Protein bands were detected by silver staining (Harris and Angal, 1989, supra). For immunoblots, gels were electrophoretically transferred to nitrocellulose sheets by semi-dry transfer at 100mA for 1.5 hours. The transfer buffer (Towbin et al., 1979, Proceedings of the National. Academy of Sciences USA. 76, 4350-4354) contained 0.1% (w/v) SDS (Shapiro et al., 1986, Journal
of Parasitology 72, 454-463).

Preparation of Antisera

Resistance was induced in Dunkin Hartley guinea pigs by repeated infestation with R. appendiculatus nymphs. When tick feeding was significantly reduced, the animal was killed and serum collected and stored at -20°C.

Antisera were also raised against denatured unfed SGE (SGED0) and SGE derived from ticks that had fed for 6 days (SGED6). The pooled SGE of unfed ticks or day 6 feeding ticks was denatured as described above. After 10% SDS-PAGE, the separating gel was homogenized at 4°C and mixed with complete Freund's adjuvant, for the primary inoculation, or incomplete Freund's adjuvant for 2 subsequent inoculations at 10 day intervals. Approximately 40μg of total protein was inoculated into each guinea pig per injection. Ten days after the third boost, the guinea pigs were killed, and serum collected and stored at -20°C.

Control serum was derived from Dunkin Hartley guinea pigs that had not been exposed to ticks or tick antigens.

Immunoblot Assays

Dot blotting was used to determine the titre of the antisera and immunoblotting was used to examine the protein profiles. One μl (1μg/μl) of denatured antigen was applied to nitrocellulose sheets at room temperature for dot blotting or electrophoretically transferred for immunoblotting. Blots were rinsed in PBS-Tween (0.05% Tween in PBS) three times, incubated in 5% non-fat dry milk in PBS-Tween at room temperature for 30 minutes to block non-specific binding, rinsed in PBS-Tween three
times, and then incubated in antisera or control guinea serum (diluted in 5% milk PBS-Tween) at room temperature for 2 hours. After rinsing three times in PBS-Tween, the blots were incubated in goat anti-guinea pig IgG alkaline phosphatase conjugate (Sigma) diluted to 1:1500 in 5% milk PBS-Tween, at room temperature for 1 hour, and then rinsed in PBS-Tween three times. The blots were finally incubated in Tris-buffered saline (100mM NaCl, 5mM MgCl₂, 100mM Tris HCl), pH 9.5, containing 0.22% NBT (nitro-blue tetrazolium) and 0.17% BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma) at room temperature to develop the colour.

RESULTS

Changes in total protein content of salivary glands, saliva and haemolymph during feeding

The total soluble salivary gland protein content increased about 12 times from 4.9 µg/tick in unfed female ticks to 60 µg/tick in engorged female ticks (Fig. 1). The greatest rate of increase in protein content occurred during the first two days of feeding, and during the last two days of feeding when the ticks were in the rapid phase of engorgement. In addition, there were detectable differences between the protein profiles of salivary glands at different stages of feeding (Fig. 2). Differences were also observed in the protein profiles of saliva collected on days 6 and 8 of feeding, and these changes differed from those observed in salivary glands (Fig. 2). Some new protein bands appeared in SGE during feeding (e.g. 23.5 Kd) and bands apparently specific for unfed ticks disappeared (e.g. 45 Kd) while some other protein bands changed their intensity during feeding (98 Kd). There were a few proteins that did not appear to change (e.g. 29 Kd). Generally, a similar phenomenon was observed in
haemolymph protein profiles (Fig. 3), with protein bands disappearing (e.g. 153 Kd), appearing (e.g. 120 Kd), and remaining unchanged (e.g. a dominant protein of 98 Kd) during the feeding period.

**Titration of antisera using dot blotting**

The titre of the serum raised against feeding nympha (tick immune serum) was > 1:1000 as determined by incubating with denatured SGED6. Titres of the antisera raised against denatured SGEDO and SGED6 were > 1:10000 ten days after the third boost.

**Comparison of proteins detected by immunoblotting**

In order to examine the changes in salivary glands, saliva and haemolymph during feeding, protein profiles were analysed by immunoblotting. Serum from guinea pigs that had not been exposed to ticks was used (1:100) as a control. When SGED6 was used as the antigen, two bands (55 and 78 Kd) were developed (Fig. 4).

The tick immune serum raised against feeding nympha was used (1:100) to detect antigens in unfed and partially fed female ticks. Proteins in saliva of day 6, SGEDO and SGED6, and haemolymph of day 0 and day 6 were detected (Fig. 5). Only five bands were recognised in saliva of day 6 (55, 72, 98 Kd and two very weak bands, 156 and 165 Kd). The haemolymph collected on days 0 and 6 shared the 98 Kd weak band, but a 55 Kd band only appeared in haemolymph of day 6. The SGEDO antigen had a stronger bottom band (<14.5 Kd) than SGED6. SGEDO also had a unique strong 23.8 Kd band while SGED6 had a unique very weak 180 Kd band. SGEDO and SGED6 shared the 48.5, 72, 78, and 85 Kd bands whereas only the 72 Kd band appeared in both the haemolymph samples and saliva of day 6.
Antiserum to denatured SGEDO (1:500) was used to detect antigens in SGE of day 0 to day 8, and saliva of day 6 and day 8 (Fig. 6). Both large and small proteins in SGE samples disappeared during feeding. SGED2 shared all of the major bands with SGEDO (<14.5, 16.5, 21.2, 82, 83, 85, 88.5, 112, 115, 160, and >200 Kd). Some weak bands (54.5, 63, and 118 Kd) were also shared by SGED2 and SGED0 but these bands were even less intense in SGED2. Two bands (59.5 and 78 Kd) were detected in all the SGEs. A few weak bands were developed in saliva of day 6 (55, 58.5, 62, 66, 70 Kd); none of them appeared in day 8 saliva. Both the 66 and 70 Kd bands were shared by SGED4 and SGED6. Haemolymph proteins also reacted with the SGED0 serum (Fig. 7). These included some large protein bands that were not developed in either SGE or saliva samples. Most of the bands detected in saliva also appeared in haemolymph samples (55, 58.5, 66, and 70 Kd).

Antiserum to denatured SGED6 (1:500) was also used to detect antigens in SGE day 0 to day 8, saliva of day 6 and day 8, and haemolymph of day 0 to day 8. All the SGEs shared three of the major bands (78, 108, and 145-147 Kd) (Fig. 8). Some weak bands (58.5, 66, 72, and 88.5 Kd) were also common to all the SGEs. Several small protein bands (15, 17.6, 20, 21.5, 22.5, 23.5, and 24 Kd) appeared in the later feeding SGEs. The common 66 and 88.5 Kd bands also appeared in the saliva although weakly, whereas the 55, 58.5, and 72 Kd bands were much stronger in saliva than in SGEs. The 62 and 70 Kd bands in saliva did not appear in SGEs. Except for the 62 Kd protein band, the bands in saliva were also detected in haemolymph samples (Fig.9). In addition, some of the small proteins (15, 21.5, 22.5, 23.5, and 24 Kd) found in SGEs also appeared in the haemolymph as feeding progressed.
SUMMARY

Tick immune serum recognized more antigens in the unfed tick SGE than in feeding ticks (day 6), and only 5 antigens were recognised in saliva of day 6 feeding ticks. The data indicate that antigens secreted by early feeding ticks have a greater influence on the host’s B cell response than those secreted by later feeding ticks. The immunoblotting data of SGEs with SGED0 antiserum (Fig. 6) also demonstrated that some proteins found in unfed tick salivary glands disappeared or were reduced in concentration significantly after attachment.

The sera raised by denatured SGEs recognized a greater number of bands compared with the tick immune serum; many were new bands that were not recognized by tick immune serum. The results confirmed that the method of using denatured antigens successfully induced antibodies against the weakly immunogenic proteins or non-secreted antigens to which naturally infested guinea pigs would not be exposed.

New proteins may be detected by immunoblotting after commencement of feeding (Fig. 8); in addition, there were some common proteins (two of the strongest bands, 78, and 108 Kd). The major bands near the top of the gel changed: in the unfed and day 2 ticks the apparent size was 147 Kd, two days later it was 146 (145-147) Kd, and then on day 6 two bands developed, 145 and 146 Kd, and on day 8 both these bands were weaker. The antiserum was raised against denatured SGED6 antigen and should rely on the primary or secondary structure of the antigens. Thus the antigens of 145-147Kd should have similar primary or secondary structural characteristics; their different molecular weights may be due to protein modification in tick salivary glands during feeding.
A common protein (98 Kd) was dominant in silver stained gels of SGEs, saliva, and haemolymph samples (Figs. 2 and 3). This protein was recognised as a weak band by the tick immune serum in addition to a 72 Kd band. Both the SGED6 and SGED0 antisera also recognized a few common bands (58.5, 66, and 70 Kd) in SGEs, saliva and haemolymph samples. In SGEs these bands were weaker than in either saliva or haemolymph with SGED6 serum. Comparison of the silver stained proteins indicated that the differences detected by immunoblotting were not due to differences in the relative concentrations of the proteins in the various samples. The fact that the common bands detected with SGED6 serum were weaker in intensity with SGED6 suggests that the SGED6 proteins are precursors of the antigenically related proteins detected in haemolymph and saliva. These observations may be further evidence that protein modification is occurring in the salivary glands, and the products may enter the haemolymph and be secreted in saliva.

Both the tick immune serum and the SGED6 antiserum recognized strong bands in SGED6, but neither of them developed similar sized bands in saliva of day 6. If the saliva proteins lost some of their antigenic characteristics during secretion, they may no longer be recognized by SGED6 antiserum. Similar reasoning may explain why the SGED0 antiserum detected comparatively few bands in later feeding SGEs (Fig. 6) whereas the SGED6 antiserum developed strong bands in unfed and early feeding SGE (Fig. 8). Alternatively, the results may indicate that the salivary gland does not secrete its products via saliva during the later period of feeding as much as during the early feeding time.

The SGED6 antiserum also recognized some small proteins (15, 21.5, 22.5, 23.5, and 24 Kd) in both SGEs and haemolymph samples from day 4 of feeding. The 21.5,
22.5, and 24 Kd bands appeared in haemolymph earlier than in SGEs: they appeared in hemolymph on day 4, increased by day 6, and decreased by D8 while they appeared in SGE on day 6 and become stronger by day 8 (Fig. 8). These proteins appear to be derived from the haemolymph and then enter and remain in the salivary glands during the later feeding period. Other small proteins (15 and 23.5 Kd) may behave similarly. Thus much of the evidence suggests that salivary glands and haemolymph not only share some common proteins but also may cooperate in some of their functions during feeding.

Shapiro et al. (1986) (supra) reported that there were salivary gland proteins nonimmunospecifically bound by antibodies from a guinea pig not exposed to R. appendiculatus ticks. The control guinea pig serum we used also recognized two salivary gland proteins (55 and 78 d). This nonspecific binding of a 55 Kd protein was also observed with some of the haemolymph and saliva samples. All of the three immune sera also recognized these two antigens. Indeed, the 78 Kd band in SGE day 0 to day 8 developed by SGED6 antiserum was much stronger than that developed by control serum. Further investigation demonstrated that the 55 Kd band was the heavy chain of guinea pig IgG (see Example 2). Although it is recognised that host immunoglobulins can pass across the gut wall into the haemolymph during tick feeding, the fate of these proteins has not previously been determined.
EXAMPLE 2

Excretion of host immunoglobulin in tick saliva and detection of IgG-binding proteins in tick haemolymph and salivary glands

MATERIALS AND METHODS

Ticks and Guinea Pigs

A laboratory colony of *R. appendiculatus* was maintained by feeding all three parasitic stages on the shaved backs of Dunkin Hartley guinea pigs. For the experiments, equal numbers of male and female ticks were fed together. When they had fed for the desired period, females were removed from the guinea pigs and rinsed in water at least three times.

Collection of Tick Samples

Samples were collected as described in Example 1. Briefly, haemolymph of unfed and partially fed ticks was collected into capillary tubes after cutting one or more legs, and then immediately pooled and diluted into cold phosphate buffered saline (PBS). Salivary gland extract (SGE) was obtained by dissecting salivary glands from either unfed or partially fed female ticks in cold PBS, rinsing in fresh PBS twice, and then homogenizing the pooled salivary glands in cold PBS. The homogenate was centrifuged at 10,000g for 10 minutes at room temperature and the supernatant collected. Extracts prepared from salivary glands of unfed ticks, and from ticks collected at 2 day intervals of feeding, were labelled SGED0, SGED2, etc. Salivation was induced by injecting female ticks parenterally with 2 mM dopamine in PBS within 1 hour of removal of female ticks from the guinea pig host. Saliva was collected in a capillary
tube placed over the chelicerae and hypostome. The concentration of total protein in the tick samples was detected using the Bio-Rad Protein Assay Kit with bovine serum albumin as the standard. All samples was stored at -20°C.

Immunoblotting with SGE

Reduced SDS-PAGE (Harris & Angal, 1989, supra) was used to separate SGE proteins with 15 μg of total protein loaded in each lane. The 12.5% resolving gel was electrophoretically transferred to a nitrocellulose sheet by semi-dry transfer at 100mA for 1.5 hours. The transfer buffer (Towbin et al., 1979, supra) contained 0.1% (w/v) SDS (Shapiro et al., 1986, supra). The blot was rinsed in PBS-Tween (0.05% Tween in PBS) three times, incubated in 5% non-fat dry milk in PBS-Tween at room temperature for 30 minutes to block non-specific binding, rinsed in PBS-Tween three times, and then incubated at room temperature for 1 hour in goat anti-guinea pig IgG alkaline phosphatase conjugate (Sigma) diluted to 1:1500 in 5% milk PBS-Tween. After rinsing three times in PBS-Tween, the blot was incubated in Tris-buffered saline (100mM NaCl, 5mM MgCl₂, 100mM Tris-HCl, pH 9.5), containing 0.22% nitro-blue tetrazolium and 0.17% 5-bromo-4-chloro-3-indolyl phosphate (Sigma) at room temperature to develop the colour. Only the conjugate was used for detecting host IgG, and guinea pig serum proteins were used as a positive control.

Enzyme-Linked Immunosorbent Assay (ELISA) to Detect the Concentration of IgG in Tick Samples

The ELISA plate was coated with 50 ng/well of goat IgG anti-guinea pig IgG (Sigma) in 100 μl of carbonate buffer (200mM Na₂CO₃, 200mM NaHCO₃, pH 9.6), and left at 4°C overnight. After washing three times with PBS-
Tween, 100 µl/well of 5% milk PBS-Tween was added to block non-specific reactions, and the plate incubated at 37°C for 30 minutes. Following three washes with PBS-Tween, 100 µl of tick sample or the standard guinea pig IgG (Sigma) (25-0.01ng/100 µl/well) diluted in 5% milk PBS-Tween were added per well and the plate incubated at 37°C for 1 hour. The plate was washed three times with PBS-Tween, and then 100 µl/well of conjugate (goat anti-guinea pig IgG linked alkaline phosphatase; Sigma) diluted in 5% milk PBS-Tween (1:3000) added. After further incubation at 37°C for 1 hour, and three washes with PBS-Tween, 100 µl/well of 0.1% p-nitrophenyl phosphate disodium (Sigma) were added and the O.D. value at 410 nm obtained. The zero value was determined using wells containing coating buffer in place of the anti-guinea pig IgG, to compensate for any non-specific binding. Replacement of the tick samples or guinea pig IgG standard with 5% milk PBS-Tween acted as a control. The amount of guinea pig IgG in each tick sample was determined from the standard curve and the relative concentration of guinea pig IgG against total protein was deduced.

**ELISA to Detect the Specific Biological Activity of IgG in Tick Samples**

Killed *Escherichia coli* emulsified with incomplete Freund's adjuvant was used to immunize guinea pigs (approximately 150 µg/animal administered in three doses). Ticks were fed on the immunized guinea pig for 6 days. Haemolymph, SGE, and saliva were collected as described above. An ELISA plate was coated with 50ng/well of killed *E. coli* and the procedures followed as described above. The zero value was determined from the wells without antigen (carbonate buffer instead of *E. coli*). Non-immunized guinea pig serum and samples of the ticks fed on a non-immunized guinea pig were used as
negative controls and a series of diluted immunized guinea pig serum on which the ticks were fed was used as the positive control.

IgG-Agarose Affinity Column for Isolation of IgG Binding Proteins

A 1 ml guinea pig IgG linked agarose (Sigma) column was used to isolate the IGBP in day 6 fed female tick samples. Haemolymph and salivary gland samples were obtained as above from female ticks at day 6 of feeding, but diluted and extracted in 0.5% of TritonX-100 in Tris-HCl buffered saline (50mM Tris, 150mM NaCl, 20mM CaCl₂, and 20mM MgCl₂, pH 7.0: TBS-Triton). The column was washed twice with 5ml TBS-Triton pH 7.0, 2 ml of glycine-HCl buffered saline (GBS: 100mM glycine, 150mM NaCl, pH 2.6), and then 5ml TBS-Triton pH 7.0. One ml of tick sample (haemolymph or SGE) in TBS-Triton pH 7.0 was loaded onto the column. A vacuum pump was used to recycle the sample through the column for 1 hour at 28°C. Then the column was washed with at least 10 ml of TBS-Triton pH 7.0, and eluted with 2ml of GBS pH 2.6. The eluted proteins were precipitated with trichloroacetic acid at a final concentration of 20%. The pellet was washed in 100% acetone and then dried. SDS-PAGE sample buffer was used to dissolve the precipitated proteins. The samples were boiled for 2 minutes and then subjected to SDS-PAGE using a 15% resolving gel. The gels were stained with Coomassie blue. The last 2 ml fraction of the TBS-Triton wash was also collected and examined by the same SDS-PAGE system to ensure that the column was washed clean before elution with GBS pH 2.6. Salivary glands extracted in PBS were examined using the same method as described above except that PBS replaced TBS-Triton.
RESULTS

Immunoblotting

The results of immunoblotting using an anti-guinea pig IgG detector and guinea pig serum as the positive control demonstrated that the 55 kD band in tick SGE was the heavy chain of guinea pig IgG (Fig. 1). No other bands were developed by the conjugate in either guinea pig serum or tick SGE.

IgG Concentration

The concentration of total IgG in tick samples (ng of IgG/µg of total protein per sample) is shown in Fig 11. Negative controls had OD values of zero. In haemolymph of unfed ticks (day 0) there was 0.04 ± 0.01 ng/µg total protein of IgG while it was undetectable in SGEDO. IgG in the haemolymph samples increased from day 0 to day 4, decreased slightly on day 6 and increased markedly by day 8 to 7.4 ± 1.0 ng/µg total protein. In SGEs, IgG increased during all of the feeding period to 0.93 ± 0.30 ng/µg protein, whereas in saliva there was a peak at day 6 when the IgG concentration stopped increasing in haemolymph. It was not possible to collect saliva from unfed and early feeding (day 2) ticks.

Biological Activity of IgG

The anti-E. coli activity of host IgG in samples of ticks fed for 6 days is shown in Fig 12. Curve A shows the anti-E. coli antibody activity of diluted antiserum and the tick samples (killed E. coli was coated on the plate). Superimposed on this curve are the O.D. values obtained with haemolymph and SGE collected on day 6 of tick feeding. Similarly, curve B shows the relative amounts of IgG at different dilutions of the antiserum
collected from the *E. coli*-immunized guinea pigs on which the ticks fed (goat anti-guinea pig IgG antibody was coated on the plate). For the haemolymph sample, the total IgG titre of $1:5.2 \pm 0.2 \times 10^4$ diluted serum (curve B) was equivalent to an anti-*E. coli* activity titre of $1:1.2 \pm 0.2 \times 10^5$ diluted serum (curve A). Thus in the haemolymph sample, approximately 43% of the host IgG still had specific antibody activity. In the SGE of day 6, the IgG titre of $1:9.6 \pm 0.6 \times 10^4$ diluted serum had antibody activity of $1:2.7 \pm 0.1 \times 10^5$ diluted serum. This indicated that about 36% of the IgG retained activity in SGE of day 6. In saliva of day 6 (not shown in Fig. 12), the IgG titre of $1:5.6 \pm 0.1 \times 10^5$ diluted serum had the activity of $1:1.5 \pm 0.5 \times 10^6$ of diluted serum determined by extrapolation of curve A; approximately 37% IgG retained biological activity in saliva.

**IgG Binding Proteins in Tick Haemolymph and Salivary Gland**

Different sized IGBPs were detected in haemolymph and SGE of day 6 fed female ticks. Lane A of Fig. 13 was the clean IgG-agarose column washed with GBS pH 2.6; the bands in this lane represent background due to the removal by washing of IgG which was linked unstably to agarose. The extra bands in lane B and C show the IGBPs from haemolymph and SGE of day 6 female ticks. In haemolymph, the IgG binding proteins were relatively large (78kD and > 100kD) compared with those in SGE (23kD and 57kD). When PBS was used for extraction instead of TBS-Triton, none of the SGE proteins bound to the IgG-agarose column specifically.

**SUMMARY**

During tick feeding, host immunoglobulin passes through the gut wall into the tick haemolymph and retains
antibody activity, with specific antibodies binding to the target epitopes of salivary glands or ovaries (Ackerman, et al., 1981, supra). We found biologically active immunoglobulin in saliva and haemolymph. Determination of the IgG concentration by weight/volume (g of IgG/ml of sample) indicated that the IgG in saliva was about 10-fold less than in haemolymph and 10,000-fold less than in guinea pig serum.

The salivary glands exclude molecules the size of inulin (5 kD) and polyethylene glycol (M_r 4000) hence it is unlikely that IgG in haemolymph diffuses out into the saliva in a nonspecific manner, or passes out nonspecifically during maintenance of the water balance by tick salivary glands. If such nonspecific mechanisms occurred (or if IgG in saliva was due to contamination from the haemolymph during collection), other haemolymph proteins would also be present in saliva and, consequently, the relative concentration of IgG in saliva would be the same or less than in haemolymph when calculated by weight of IgG/g of total protein. This was not the case as Fig. 11 showed the relative amount of IgG in saliva was >10 times that in haemolymph on day 4 and day 6. Furthermore, the protein profiles of saliva and haemolymph at day 6 of feeding were quite different (Example 1). Thus the data are not consistent with leakage of immunoglobulins from the haemolymph through the salivary glands and into tick saliva.

The results of Fig. 11 also showed that there was detectable IgG in unfed tick haemolymph.

During the slow feeding period (before day 6), the concentration of IgG in haemolymph and SGE increased relatively slowly; in the haemolymph the concentration fell during day 4 to day 6 (Fig. 11). It appeared that the concentration of IgG within the tick was being
maintained as the bloodmeal size increased. By comparison, an increasingly high concentration of IgG appeared in tick saliva during day 4 to day 6 of feeding. The time and rate of appearance of IgG in the haemolymph, salivary glands and saliva are consistent with the hypothesis that host immunoglobulin that had passed through the gut wall was excreted from the tick body in saliva. During the period of rapid engorgement of day 6 to day 8, when a large amount of blood was taken up, the IgG concentration increased sharply in both the haemolymph and SGE. At day 8, IgG in saliva remained at a comparatively high level but was less than at day 6. This decrease may have been due to degeneration of the salivary glands at the later stages of feeding.

The IgG in haemolymph, SGE, and saliva of day 6 feeding ticks retained 35-42% of the biological activity of host serum IgG. Thus after passing through the gut wall, the IgG molecules were not apparently subjected to further significant breakdown. If the host on which the tick fed had acquired immunity to the tick, specific antibodies entering the haemocoel might damage the tick. Similarly, if the host was immune to an agent infecting the tick, specific antibodies could bind to antigens of the agent within the tick. Such antibody binding to foreign proteins in the tick might also cause damage, particularly if the infecting agent was a virus such as Thogoto virus which expresses viral antigens on the outer membranes of the tick synganglion. Thus there is strong selective pressure on the tick to get rid of host immunoglobulins. In the apparent absence of a mechanism to breakdown IgG within the haemocoel, our observations indicate that the tick has a mechanism for eliminating host immunoglobulin via saliva.
The discovery of IGBPs in haemolymph and salivary glands provides an explanation of how the tick can excrete host immunoglobulin in saliva during feeding. When host immunoglobulin passes through the tick gut wall into the haemolymph, the IGBPs in haemolymph may recognize and bind or react with them. Then the haemolymph IGBPs may transport the host IgG to the salivary gland where the salivary gland IGBPs take over and bind to the IgG. By some unknown mechanism, the host IgG is finally excreted in saliva. This system of IgG transport could represent a mechanism to protect the tick against the potentially harmful effects of immunoglobulins from immune hosts.

EXAMPLE 3

Experiments to show that ticks possess immunoglobulin-binding proteins (IGBPs)

METHODS

Collection of Tick Haemolymph. Haemolymph was collected into capillary tubes after cutting one or more legs (Ben-Yakir et al., 1986, (supra)) and immediately pooled and diluted into cold 0.5 % of TritonX-100 in Tris-HCl buffered saline (TBS-Triton: 50mM Tris, 150mM NaCl, 20mM CaCl₂, and 20mM MgCl₂, pH 7.0). The diluted haemolymph was then denatured in SDS-PAGE sample buffer (5% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, and 0.0625 mM Tris-HCl, pH 6.8) by boiling for 2 minutes, and stored at -20°C before use.

Preparation of Salivary Gland Extracts (SGE). Salivary glands were dissected from ticks in cold TBS, rinsed in fresh TBS, and then pooled and homogenized in TBS-Triton. The homogenate was centrifuged at 10,000g for 10 minutes at room temperature and the concentration of
total proteins in the supernatant fluid determined. This SGE was denatured by boiling for 2 minutes in SDS-PAGE sample buffer and stored at -20°C. Extracts prepared from salivary glands of unfed ticks, and from ticks collected after 6 days of feeding, were labelled SGE0 and SGE6, respectively.

**IgG-Agarose Affinity Column for Isolation of IgG Binding Proteins (IGBPs).** A 1 ml guinea pig IgG linked agarose (Sigma) column was used to isolate the IGBPs in tick samples. Haemolymph and SGE were obtained, as described above, in 0.5 % of TBS-Triton. The column was washed twice with 5ml TBS-Triton pH 7.0, 2 ml of glycine-HCl buffered saline (GBS: 100mM glycine, 150mM NaCl, pH 2.6), and then 5ml TBS-Triton pH 7.0. One ml of tick sample (haemolymph or SGE) in TBS-Triton pH 7.0 was loaded onto the column. A vacuum pump was used to recycle the sample through the column for 1 hour at 28°C. The column was then washed with at least 10 ml of TBS-Triton pH 7.0, and eluted with 2ml of GBS pH 2.6. The eluted proteins were precipitated with trichloroacetic acid at a final concentration of 20%. The pellet was washed in 100% acetone and then dried. SDS-PAGE sample buffer was used to dissolve the precipitated proteins. The samples were boiled for 2 minutes and then subjected to SDS-PAGE using a 15% resolving gel. The gels were stained with Coomassie blue. The last 2 ml fraction of TBS-Triton was also collected and examined by the same SDS-PAGE system to ensure that the column was washed clean before elution with GBS pH 2.6. A 1 ml agarose-6B column was also used to check the procedure using tick samples to make sure that none of the tick proteins were retained by the agarose column when IgG was absent.

**Preparation of Antisera.** Antisera were raised against denatured IGBP from 600 male *Rhipicephalus*
appendiculatus ticks that had fed for 6 days. IGBPs were separated by SDS-PAGE (15% resolving gel) after isolation using a 5 ml affinity column with the same protocol as described above. Individual IGBP bands were cut out from the separating gel and homogenized at 4°C and mixed with an equal volume of ISA adjuvant (Montanide ISA 50, Seppic, France) for the primary inoculation, and 2 subsequent inoculations at 10 day intervals. Ten days after the third boost, the guinea pigs were humanely killed, and serum collected and stored at -20°C.

Control serum was derived from Dunkin Hartley guinea pigs that had not been exposed to ticks or tick antigens.

**Immunoblotting with SGE.** Reduced SDS-PAGE (Harris & Angal, 1989, (supra)) was used to separate SGE proteins with 15 µg of total protein loaded in each lane. The 15% resolving gel was electrophoretically transferred to a nitrocellulose sheet by semi-dry transfer at 100mA for 1.5 hours. The transfer buffer (Towbin et al., 1979) contained 0.1% (w/v) SDS (Shapiro et al., 1986). The blot was rinsed in PBS-Tween (0.05% Tween in PBS: 10mM phosphate, 140mM NaCl, pH 7.3) three times, incubated in 5% nonfat dry milk in PBS-Tween at room temperature for 30 minutes to block non-specific binding, and then rinsed in PBS-Tween three times. The blot was then rinsed in 50mM NaAcetate buffer (pH 4.5) once, incubated in 5mM periodic acid in NaAcetate buffer for 1 hour at room temperature in the dark, washed in NaAcetate buffer three times, and then incubated in 1% of glycine in PBS-Tween at room temperature for 30 minutes. After washing in PBS-Tween three times, the blot was incubated at room temperature for 1 hour in antiserum which was diluted in 5% milk-PBS-Tween (1:200), washed in PBS-Tween three times, and then incubated at room temperature for 1 hour.
in goat anti-guinea pig IgG alkaline phosphatase conjugate (Sigma) diluted to 1:1500 in 5% milk-PBS-Tween. After rinsing three times in PBS-Tween, the blot was incubated in Tris-buffered saline (100mM NaCl, 5mM MgCl₂, 100mM Tris-HCl, pH 9.5), containing 0.22% NBT (nitro-blue tetrazolium) and 0.17% BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma) at room temperature to develop the colour.

RESULTS

(1) **IGBP in salivary glands of unfed male and female R. appendiculatus**

SDS-PAGE analysis as shown in Figs. 14 and 15 shows that IGBPs detected in the salivary gland extracts of unfed male and female ticks have similar MW: 56, 53, 21.7, and 20.8 kD. The ticks were examined >6 months after moulting.

(2) **IGBP in salivary glands of female R. appendiculatus after 6 days of feeding**

As can be seen from Fig 16, day 6 female ticks have the same sized IGBPs as unfed ticks, and several additional bands in the gel.

(3) **IGBP in salivary glands of male R. appendiculatus after 6 days of feeding**

As can be seen from Fig. 17, male day 6 ticks have IGBPs that differ in size from those of either unfed ticks or day 6 female ticks. Day 6 male ticks have 3 dominant IGBPs in their salivary glands (29-30 kD: IGBP MA; 23-25 kD: IGBP MB; 19-21 kD: IGBP MC). The amount of IGBP MC is much greater than that of any other IGBP.
(4) **IGBPs in salivary gland of other tick species**

Salivary glands of two other (unfed) tick species (Amblyomma variegatum female and Ixodes hexagonus males and females) were examined for IGBPs. As can be seen from Fig. 18, A. variegatum has a 49 kD and three small (14-16 kD) IGBPs, and the Ixodes tick has a 46 kD and a 15 kD IGBP.

(5) **IGBP in haemolymph of R. appendiculatus**

Haemolymph of unfed and day 6 male and female ticks was also examined for IGBPs. Protein bands of 98 kD and 87 kD were found in the haemolymph samples of unfed and fed male and unfed female ticks. An additional band of 78 kD IGBP was found in the haemolymph of females collected at day 6 of feeding (Fig. 19).

(6) **The IGBPs in day 6 male R. appendiculatus also bind to IgG of other vertebrate species**

As can be seen from Fig. 17, all the IGBPs (MA, MB, and MC) that bind to guinea pig IgG also bind to human IgG, but only IGBP MC (19-21 kD) binds to bovine IgG.

(7) **Amino acid sequence of male IGBPs**

N-terminal sequencing studies have been carried out on the male R. appendiculatus IGBPs and the following results were obtained. (Residues in lower case are the best guess)

- IGBP MA: YEVYTGRTTVTVNQYEIPADV
- IGBP MB: TFAIVAVLGSLGGPPEAPQYHPMLR-DTDhsa
- IGBP MC: AVQHYQLIK-SQ-YSAEIMG

Comparison with the SwissProt database revealed an interesting low level homology of IGBP MB (26 kD) with fibrinogen-like protein A precursor. However more
sequence data is needed before significant comparisons can be made.

In addition, cDNAs have been obtained from mRNA of male *R. appendiculatus* salivary gland IGBPs. It appears from these studies that two of the proteins, namely the 21 kD and 25 kD IGBPs (MB and MC) may be related. An alignment of the amino acid sequences is shown in Fig. 20.

(8) **Antisera raised against male IGBP**

SDS-PAGE purified IGBP MA, MB, and MC were used to hyperimmunise guinea pigs. After the third inoculation, serum of guinea pig 523 and 524 developed the IGBP MC band specifically in both purified antigen and total salivary gland proteins using immunoblotting. Serum of guinea pig 525 recognized IGBP MA and MB, and guinea pig 526 serum reacted with IGBP MA. The results can be seen in Fig. 21.

**EXAMPLE 4**

*Molecular weights of IGBPs in the haemolymph and salivary glands of various ixodid tick species*

The sizes of the various IGBPs detected in the different stages of the three ixodid tick species as determined using the protocol of Example 3 are summarised in Table 1.
Table 1. Immunoglobulin-G binding proteins in haemolymph and salivary gland extract of ixodid ticks.

<table>
<thead>
<tr>
<th></th>
<th>R. appendiculatus</th>
<th>A. variegatum</th>
<th>I. hexagonus</th>
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<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>H0(^1)</td>
<td>H6</td>
<td>SGE0</td>
<td>SGB6</td>
</tr>
<tr>
<td>98kD(^2)</td>
<td>98kD</td>
<td>54kD</td>
<td>54kD</td>
</tr>
<tr>
<td>88kD</td>
<td>88kD</td>
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\(^1\) H0 and H6, haemolymph collected from unfed ticks and at day 6 of feeding, respectively; SGE0 and SGB6, salivary gland extract of unfed ticks and ticks at day 6 of feeding, respectively.

\(^2\) Relative molecular weights estimated on SDS-PAGE. Values for *R. appendiculatus* are the means of 3 samples; values for the other samples were determined using one preparation for each.

\(^3\) Three proteins in the range 16.5 to 14.5 kD appear as a single fuzzy band.
EXAMPLE 5

Immunoglobulin G binding proteins in male Rhipicephalus appendiculatus ticks

Ticks and Guinea Pigs. Ticks were maintained as described in Example 2. For determining whether male IGBPs were affected by copulation, male ticks were fed with or without female ticks. In all other experiments, equal numbers of male and female ticks were fed together.

Collection of Tick Samples. Samples were collected and salivary gland extract prepared as described in Example 3. The concentration of total protein in the tick samples was detected using the Bio-Rad Protein Assay Kit with bovine serum albumin (dissolved in TBS-Triton at 1 mg/ml) as the standard. All samples for SDS-PAGE or immunoblotting were denatured in SDS-PAGE sample buffer (reducing conditions) at 100°C for 2 minutes, then stored at -20°C. For isolation of IGBP the SGE was used immediately.

IgG-Agarose Affinity Column for Isolation of IgG Binding Proteins. A one ml guinea pig IgG linked-agarose (Sigma) column was used to isolate the IGBP in SGE of male ticks exposed to guinea pigs for 6 days, using the method described in Example 3. Human and bovine IgG-agarose (Sigma) columns were used, in the same manner as guinea pig IgG-agarose, to determine the ability of IGBPs to bind to IgG of different species. Salivary glands from 10 male ticks were used for each column.

Preparation of Antisera. Antisera were raised against denatured IGBP isolated from 600 male R. appendiculatus ticks that had infested guinea pigs for 6 days as described in Example 3. Guinea pigs numbered 523, 525
and 526, were immunized with IGBP-MC, -MB and -MA, respectively.

**Immunoblotting with SGE.** Immunoblotting procedures were as described previously in Example 3 with the following modifications. The blot was probed for 2 hours with anti-IGBP sera diluted 1:100 in 5% milk PBS-Tween, washed three times with PBS-Tween and incubated at room temperature for 1 hour in goat anti-guinea pig IgG alkaline phosphatase conjugate (Sigma) diluted 1:3000 in 5% milk PBS-Tween. The signal was generated as described previously.

**RESULTS**

Using a guinea pig IgG-agarose column and SDS-PAGE, three abundant IGBPs were isolated from SGE of day 6 male *R. appendiculatus* (Fig. 22). The molecular weights of the three IGBPs measured by SDS-PAGE were: IGBP-MA, 29 kD; IGBP-MB, 25 kD; IGBP-MC, 21 kD. One smaller and several larger but less abundant proteins appeared to be isolated by the procedure, including a 54 kD tick protein observed previously and the 55 kD heavy chain of guinea pig IgG. Anti-IGBP sera were obtained using SDS-PAGE purified antigens. When immunoblotting was performed with the total protein of SGE from day 6 male *R. appendiculatus*, serum of guinea pig (GP) 523 was specific for IGBP-MC, GP 526 serum reacted with IGBP-MA, and GP 525 serum reacted with IGBP-MB and -MA (Fig. 23). The results shown in this Figure suggest that the denatured IGBP-MA, -MB and -MC are not antigenically related. Periodic acid treatment to remove saccharides from the proteins markedly reduced the background due to cross-reactions with other SGE proteins (Fig. 23, lanes 2 to 4). This suggests that some of the IGBPs may be glycoproteins.
When the guinea pig IgG-agarose was replaced by either human or bovine IgG-agarose, the three IGBPs showed differences in their binding abilities (Fig. 24). IGBP-MC bound to IgG of all three species whereas IGBP-MA did not bind to bovine IgG. It was not clear whether IGBP-MB could bind to human or bovine IgG because it migrated in the gels in the same position as the light chain of the respective IgG. The human IgG sample (Fig. 24, lane 5) bound many more tick proteins compared with the guinea pig and bovine samples, even though the last washing fraction was clean (Fig. 24, lane 6).

The protein profiles of male tick salivary glands were compared with those of female ticks over an 8 day infestation period (Fig. 25). The profile of female tick salivary glands changed during feeding. For unfed ticks, the SGE protein profile was similar for males and females. Thereafter, the profile of male SGE changed, and the changes differed from those observed in female SGE. When examined by immunoblotting using antisera raised against IGBP-MA, -MB and -MC sera, all three IGBPs were shown to be male specific (Fig. 26). The IGBPs were first detected in SGE of day 4 male ticks; their appearance was independent of whether or not the male ticks fed with females (data not shown). The apparent molecular weight of IGBP-MA and IGBP-MC remained unchanged from day 4 to day 8 whereas the diffuse band of IGBP-MB (possibly a doublet) appeared to have a slightly higher molecular weight at day 4 compared with days 6 and 8. The heavy chain of guinea pig IgG (55 kD) was detected in SGEs of male ticks but was not apparent in the comparable amount of total protein of female SGE though previously IgG had been detected when female SGED8 was extracted in PBS. The apparent change in molecular weight of IGBP-MB (Fig. 26) indicates that protein modification may occur in male salivary glands during feeding.
EXAMPLE 6

Cloning and sequencing of IGBP MA, MB and MC from R. appendiculatus

MATERIALS AND METHODS

Ticks and guinea pigs. Ticks were maintained as described in Example 2. Equal numbers of male and female ticks were fed together to encourage the feeding of male ticks.

Preparation of salivary gland extracts (SGE). Salivary glands were dissected from 100 male ticks collected after 6 days infestation and extracts prepared as described in Example 3. The SGE was used for IGBP isolation immediately.

IgG-agarose affinity column for isolation of IgG binding proteins (IGBPs). Affinity chromatography was performed as described in Example 3. The separated IGBPs were electrophoretically transferred to a nitrocellulose sheet, and stained by Coomassie blue. Individual IGBP bands were cut out for N-terminal sequencing (performed by Mr A.C. Willis, Department of Biochemistry, University of Oxford).

Immunoblotting with SGE. Immunoblotting procedures were as described in Example 5.

cDNA library construction. Salivary glands were excised from 20 male adult R. appendiculatus ticks that had been feeding on guinea pigs for six days. The glands were collected in an eppendorf tube on dry ice. mRNA was isolated using the FastTrack mRNA isolation kit (Invitrogen). For synthesis of cDNA and its unidirectional insertion into the Lambda Zap II vector,
we used the ZAP cDNA synthesis kit (Stratagene). Prior to insertion into the lambda vector, the cDNA was fractionated over a Sephacryl S-400 (Pharmacia) column. A library (termed MD6-I) was constructed with low molecular weight cDNAs (ranging from approximately 100 to 2,000 basepairs). The higher molecular weight fraction was used to construct a second library (MD6-II). Packaging was performed with Packagene (Promega) packaging extracts. Approximately $1.5 \times 10^6$ plaque-forming units (PFU) of each library were amplified in XL-1-Blue cells (Stratagene).

**Screening a phage cDNA library using specific antisera.**

This was undertaken by *in vivo* excision of the pBluescript SK(-) phagemids from a fraction of the library using the RU408 helper phage (Stratagene) (Short et al., 1988, Nucl. Acids Res. 16, 7583-7600). Five µl of the MD6 phage library was diluted 1:500 in SM buffer (5.8g NaCl, 2.0g MgSO4, 50ml 1M Tris-HCl, pH 7.5, 5ml 2% gelatin per litre; autoclaved) and then added to 200µl XL-1-Blue cells (adjusted to O.D$_{600}$=0.5 in 10mM MgSO$_4$). The culture was incubated shaking at 37°C for 15 minutes then mixed with 3ml of prewarmed (50°C) top agar (NZY broth: 5g NaCl, 2g MgSO$_4$.7H$_2$O, 5g yeast extract, 10g NZ amine, pH 7.5, 7g agarose, per litre; autoclaved). The top agar was poured into 90cm$^2$ NZY plates (11 NZY broth mixed with 15g agar; autoclaved). Plates were incubated at 37°C until phage plaques became visible. A nitrocellulose filter (Gelman Sciences) was wetted in 10mM IPTG (isopropyl-β-D-thiogalactopyranoside) and air dried at room temperature. The nitrocellulose filter was placed on the plate which was then incubated at 37°C for 4 hours. The filter was removed from the plate, and the blot rinsed in PBS-Tween (0.05% Tween in PBS: 10mM phosphate, 140mM NaCl, pH 7.3) three times and then incubated in 5% non-fat dry milk in PBS-Tween at room temperature for 30 minutes to block non-specific
binding. After washing in PBS-Tween three times, the blot was incubated at room temperature for 1 hour in antiserum which was diluted in 5% milk-PBS-Tween (1:200), washed in PBS-Tween three times, and then incubated at room temperature for 1 hour in goat anti-guinea pig IgG alkaline phosphatase conjugate (Sigma) diluted to 1:1500 in 5% milk-PBS-Tween. After rinsing three times in PBS-Tween, the blot was incubated in Tris-buffered saline (100mM NaCl, 5mM MgCl₂, 100mM Tris-HCl, pH 9.5), containing 0.22% NBT and 0.17% BCIP at room temperature to develop the colour. Positive clone were picked and diluted in SM buffer. Selections were repeated until a positive clone was isolated.

**In vivo excision.** Positive plaques were incubated in 500µl SM buffer and 20µl chloroform at 4°C overnight to release the phage from bacterial cells. Then 200µl of the phage stock was mixed with 200µl of XL-1-Blue cells (O.D.₆₀₀=1.0) and 1µl R408 helper phage (>1x10⁶ pfu/ml; Stratagene). The mixture was incubated shaking at 37°C for 15 minutes. Five ml of 2XYT medium was added and incubated shaking at 37°C for 3 hours. The mixture was held at 70°C for 20 minutes and then centrifuged for 5 minutes at 4000g. One µl of the supernatant containing the rescued phagemid was mixed with 200 µl of XL-1-Blue cells (O.D.₆₀₀=1.0), and incubated at 37°C for 15 minutes with shaking. 100 µl aliquots of the mixture were plated on LB/ampicillin plates and incubated overnight at 37°C.

"Speedprep" purification of plasmids. As described by Goode and Feinstein (Goode & Feinstein, 1992, BioTechniques 12, 374-375), the XL-1-Blue E. coli cells containing relevant inserts were grown overnight shaking in liquid LB/ampicillin medium at 37°C. 1.5 ml of the culture was centrifuged at top speed for 30s and the pellet resuspended in 100 µl solution A (50 mM Tris-HCl,
pH 8.0, 4% Triton X-100, 2.5 M LiCl, 62.5 mM EDTA). 100 μl of a Tris-HCl-buffered phenol/chloroform mixture (1:1) was added, and the mixture vortexed for 10 s and then centrifuged at top speed for 2 minutes. The water phase was removed to a fresh eppendorf tube containing 200 μl cold 100% ethanol, vortexed briefly, centrifuged for 5 minutes, and the pellet then washed with 1 ml 70% ethanol and dried. The plasmid pellet was resuspended in 10 μl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 100 μg/ml RNase A, and incubated at room temperature for 5-20 minutes.

Constructing a plasmid library. A mixture comprising 200 μl of male *R. appendiculatus* tick phage library diluted 1:500, 30 μl of R408 helper phage, and 2 ml XL-1-Blue cells (O.D.₆₀₀=1.0) was incubated at 37°C for 15 minutes with shaking. After the addition of 30 ml 2XYT medium and incubation shaking at 37°C for 3 hours, the mixture was held at 70°C for 20 minutes and then centrifuged at 4000g for 5 minutes. Twenty μl of the supernatant was mixed with 2 ml XL-1-Blue cells (O.D.₆₀₀=1.0) which were then incubated shaking at 37°C for 2.5 hours. After centrifuging the cells, the plasmids were purified by "Speedprep".

Screening the plasmid library by PCR. The plasmid library was digested using XbaI or BamHI. Selected DNA fragments (3200 to 4200 bp) were extracted from a 1% agarose-TAE (0.04 M Tris-acetate, 0.001 M EDTA) gel. Degenerate 5'-end primers were synthesized, based on the amino terminal sequence of IGBP MB and IGBP MC, as follows.

Amino acid sequence of IGBP MB:

T F A I V A V
Degenerate primers for IGBP MB:

T T T T T T
5' ACCTTGCCCATCGTCGCCGT 3'
A A A A A
G G G G G

Amino acid sequence of IGBP MC:
Q Y H Q L I K

Degenerate primers for IGBP MC:

A T T AT T T
IGBP MC 5' CAGTACCCACGCACATCAA 3'
A G
G

The 3'-end primer for PCR was the T₇ sequencing primer specific for the plasmid. A PCR (Bioline PCR kit) of 30 cycles was used to amplify the plasmid insert with an annealing temperature of 48°C.

Cloning the PCR product for sequencing. The PCR products were cloned into the pGEM-T vector (Promega) and sequenced using Sequenase with the SP6 promoter from the 5' end. The nucleotide sequence was then translated and the amino acid sequence confirmed by comparison with the N-terminal sequence obtained by amino acid sequencing of the tick IGBP proteins.

Nonradioactive DNA labelling. Labelling was performed using the DIG kit (Boehringer Mannheim Biochemica). The pGEM-T plasmid was subjected to the Speedprep procedure and then cut using XhoI for IGBP MA, EcoRI and NotI for IGBP MB, and NotI and ClaI for IGBP MC. Following gel fractionation, the insert was phenol-chloroform extracted, ethanol precipitated, and resuspended in 15 μl TE (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA was
labelled with hexanucleotides using the Klenow enzyme. Labelling was confirmed by dot blot using anti-dig-alkaline phosphatase.

**Screening the phage library for complete inserts using labelled DNA probes.** The male *R. appendiculatus* lambda library was grown up as described above for screening with antisera. Phage plaques were transferred to nitrocellulose filters without treatment with IPTG. Filters were dried at room temperature, and the DNA denatured, neutralized, and fixed onto the filter by incubation at 80°C for 2 hours. Hybridization was carried out overnight at 68°C with the dig-labelled probe. The filter was then incubated with the anti-dig-conjugate. Selections were repeated until a positive clone was isolated. Sequencing of the phage inserts was undertaken as described above.

**Sequencing.** The plasmid from one "Speedprep" was alkali-denatured (Mierendorf and Pfeffer, 1987, Methods Enzymol. 152, 556-562) in 2 μl of 2 M NaOH, 2 mM EDTA at room temperature for 5 minutes, and neutralized with 2 μl of 2 M ammonium acetate, pH 4.6: 78 μl absolute ethanol was added and the mixture incubated at -70°C for 20 minutes. After centrifugation for 10 minutes, the pellet was washed with 200 μl cold 75% ethanol, centrifuged for 1 minute, and the pellet dried. Both strands of the inserts in the purified plasmids were sequenced by means of the Sanger dideoxy-mediated chain termination reaction using Sequenase (UCB). Sequence data were analysed using the GCG sequence analysis software (Genetics Computer Group, 1991). Protein database searches were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service.
RESULTS

The amino acid sequence of the N-terminal regions of the IGBP$s$ MA, MB and MC were determined and are shown in Table 2. Screening with the antisera of guinea pig 523, 525 and 526 that were raised against IGBP MC; MB contaminated with MA; and MA, respectively, identified only IGBP MA positive clones in the phage library. Single cDNA products were obtained for IGBP MB and IGBP MC by PCR amplification of the small plasmid library using synthesized oligonucleotide primers. Full length cDNA clones for all three IGBP proteins were isolated by screening the phage library using DIG-labelled DNA probes. The DNA inserts were sequenced in both directions by subcloning the restriction enzyme digested cDNA or using synthesized sequencing primers. The nucleotide sequences were translated to amino acid sequences using the GCG program (Fig. 27A to 27C). The protein N-terminal sequences confirmed that all of the cDNAs isolated from the library were correct.

All of the three clones had ATG as start codons, and polyA tails at the end with an AATAAA signal before the polyA. Sigcleave of the GCG program indicated that all of the three IGBP proteins had signal sequences (Fig. 28). The signal sequence of IGBP MC was exactly before the first amino acid of the N-terminal. The signal sequence of IGBP MA gave a low score and did not start from the first amino acid of the real protein. The purified tick-derived IGBP MB had a leading sequence at the N-terminus. Amidation sites were identified in IGBP MA and MC using the Motifs program. The amidation site in IGBP MA was 23: SQAY NGKK YEYIT, exactly before the first amino acid of the N-terminus (YEYIT). The amidation site in IGBP MC was 148: LMLES KGKK LSCQA. Motifs also identified a N-glycosylation site in IGBP MC at 33: IKCSQ NYSA EIMGV, and three N-glycosylation sites in IGBPMB at 48: KEAVV NSTM KAKIN, 100: CKPNK NTTN
LLGNG; and 107: TMLLG NGTQ ANNGA. IGBP MB and IGBP MC were related proteins (Fig. 29). Bestfit gave the two proteins 51% identity and 70% similarity. IGBP MA did not show a significant sequence relationship with either IGBP MB or MC. Using the Blast network service, no meaningful results were obtained by searching the Gene Bank data base with the sequences of the three tick IGBPs.

EXAMPLE 7

Presence of IGBPs in male R. appendiculatus ticks after interrupted feeding

MATERIALS AND METHODS

The methodology used in Example 6 was employed to determine the effect of interrupted feeding. For the detection of IGBPs in interrupted feeding ticks by Western blotting, males were removed from guinea pigs and then maintained at 27°C and relative humidity 80% for a further 7 days before salivary glands were dissected.

RESULTS

The feeding of male ticks was interrupted to determine the effect on the protein profiles of the abundant IGBPs (Fig. 30). When male R. appendiculatus ticks were stopped from feeding for 7 days, their protein profiles of SGE differed from those of continuously-fed ticks. For example, the 22.5kD protein in D2+7, 30kD in D4+7, D6+7 and D8+7. The IGBPs profile also changed when continuous feeding was stopped (Fig. 31). A 30kD band appeared in SGE of D4+7, D6+7 and D8+7. The molecular weight of IGBP MB became slightly smaller in D4+7 from D4. The IGBPs in D2 were not detectable, but all of the
IGBP bands appeared in D2+7. The amount of IGBP MC increased in D4+7 compared with D4. Relative amounts of IGBP MB and MC decreased in D8+7 compared with D8. Fig. 32 shows the changes of individual IGBPs when tick feeding was stopped on day 6. The 30kD band was developed by the anti-IGBP MA serum (guinea pig 526) in D6+7, but not in D6. There were four smaller bands developed by this serum (23-27kD) in both lanes. The smaller bands in D6+7 were much stronger than those in D6. IGBP MB did not change between D6 and D6+7. Because the anti-IGBP MB serum (guinea pig 525) also contained antibodies against IGBP MA the difference between lane 3 and lane 4 should be due to the changing of IGBP MA. IGBP MC also did not change after the feeding had been stopped. The lower molecular weight bands developed by anti-IGBP MC serum (guinea pig 523) could be degradation products or due to a cross reaction by the serum.

Table 2. Amino acid sequences determined by direct sequencing of the N-terminal regions of IGBPs purified from male tick salivary glands

<table>
<thead>
<tr>
<th>IGBP</th>
<th>N-terminal sequence¹</th>
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<tr>
<td>MA</td>
<td>YEVTGRGVTITVDNTQYEIPADLeK</td>
</tr>
<tr>
<td>MB</td>
<td>TFAIVAVLLGSGPPEAPQYHPLR-DTDhsa</td>
</tr>
<tr>
<td>MC</td>
<td>AVQYHQLIK-SQ-YSAEIMG</td>
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¹ Amino acids indicated in lower case could not be confirmed; 
-, amino acids that could not be determined.
EXAMPLE 8

Antigenicity of GST expressed GST-IGBP MB fusion protein and Baculovirus expressed IGBP MC

MATERIALS AND METHODS

GST expressed GST-IGBP MB fusion protein and Baculovirus expressed IGBP MC were expressed as described in Example 6. The recombinant proteins were insoluble. Cell lysates were extracted in TBS-Triton (as described in Example 3) and centrifuged at 10000g for 10 minutes. The pellets were dissolved in SDS-PAGE sample buffer by boiling for 2 minutes. After electrophoresis on a 15% SDS-PAGE gel, the bands of interest were excised and used to raise antisera as described in Example 3. Western Blotting was performed as described previously.

RESULTS

Anti-GST-IGBP MB sera (GP571 and GP572) reacted strongly with tick IGBP MB in salivary gland proteins of day 6 fed male ticks and with fusion protein GST-IGBP MB in E. coli (XL-1-Blue) lysates. No non-specific cross-reaction to other male salivary gland proteins was detected.

Anti-IGBP MC sera (GP561 and GP562) reacted with IGBP MC in salivary gland proteins of day 6 fed male ticks and in insect cell (SF21) lysates. Two minor bands of lower molecular weight were also identified by the sera, which may correspond to degraded IGBP MC. Non-glycosylated IGBP MC of lower molecular weight was also identified by the sera in SF21 cell lysates.
**Claims:**

1. A protective metazoan parasite antigen, characterised in that said antigen is a protein capable of binding to host immunoglobulin, or a functionally-equivalent variant, or antigenic fragment of precursor thereof.

2. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in any one of claims 1 to 3, obtainable from helminths or arthropod parasites.

3. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 2, wherein the arthropod is an ectoparasite.

4. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 3, wherein the ectoparasite is a blood-feeding insect or acarine parasite.

5. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 4, wherein the acarine parasite is a tick.

6. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 5, wherein the tick is of the genus *Rhipicephalus*, *Amblyomma* or *Ixodes*.

7. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 6 wherein the tick is *Rhipicephalus appendiculatus*. 
8. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 7 having a molecular weight of 21, 25 or 29kD under reducing SDS-PAGE.

9. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in claim 8, comprising an amino acid sequence substantially corresponding to all or a portion of the sequence shown in any one of Figures 27A to 27C.

10. An antibody, or antigen-binding fragment thereof, which is capable of selectively binding to an antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as defined in any one of claims 1 to 9, or to the idiotype of a said antigen-binding antibody.

11. An antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in any one of claims 1 to 9 or antibody, or antigen-binding fragment thereof as defined in claim 10, for use in stimulating an immune response against metazoan parasites in a human or non-human animal.

12. Use of a metazoan parasite antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in any one of claims 1 to 9 or antibody, or antigen-binding fragment thereof as defined in claim 10, for the preparation of a vaccine composition for use in stimulating an immune response against metazoan parasites in a human or non-human animal.

13. A vaccine composition for stimulating an immune response against metazoan parasites in a human or non-human animal comprising one or more antigens,
functionally-equivalent variants, antigenic fragments or precursors thereof as claimed in any one of claims 1 to 9 or antibody, or antigen-binding fragment thereof as defined in claim 10, together with a pharmaceutically acceptable carrier or diluent.

14. A method of stimulating an immune response against metazoan parasites in a human or non-human animal, comprising administering to said animal a vaccine composition as defined in claim 13.

15. A process for the preparation of an antigen as claimed in any one of claims 1 to 9 which comprises the steps of subjecting a extract of a metazoan parasite to affinity chromatography using an immobilized phase including a specific binding partner for the antigen and subsequently eluting said antigen from said immobilized phase.

16. A process as claimed in claim 15 wherein the specific binding partner is a host immunoglobulin.

17. A process as claimed in claim 15 or 16 wherein the extract is prepared from the haemolymph, saliva or salivary glands of the metazoan parasite.

18. An antigen, use, composition, method or process as defined in any one of claims 1 to 17 wherein the animal is mammalian.

19. An antigen, use, composition, method or process as defined in claim 18 wherein the mammal is a ruminant.

20. An antigen, use, composition, method or process as defined in claim 19 wherein the ruminant is a sheep, cow, pig, deer or goat.
21. An antigen, use, composition, method or process as defined in claim 18 wherein the mammal is a companion animal.

22. A nucleic acid molecule comprising a nucleotide sequence encoding an antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in any one of claims 1 to 9 or a sequence which is degenerate or substantially homologous with or which hybridises with said sequence.

23. A nucleic acid molecule as claimed in claim 22 which comprises one or more nucleotide sequences substantially corresponding to all or a portion of the nucleotide sequence shown in any one of Figures 27A to 27C or their degenerate or allelic variants, and fragments thereof.

24. An expression or cloning vector comprising a nucleic acid molecule as claimed in claim 22 or 23.

25. A method for preparing a recombinant nucleic acid molecule as claimed in claim 24, comprising inserting a nucleotide sequence as claimed in claim 22 or 23 into vector DNA.

26. A host cell or a transgenic organism containing a nucleic acid molecule as defined in claim 22 or 23.

27. A method for preparing an antigen, functionally-equivalent variant, antigenic fragment or precursor thereof as claimed in any one of claims 1 to 9, which comprises culturing a host cell containing a nucleic acid molecule encoding all or a portion of said antigen or precursor thereof, under conditions whereby said antigen is expressed and recovering said antigen thus produced.
28. A polypeptide produced by the method as defined in claim 27.
FIG. 1

TOTAL PROTEIN CONCENTRATION (μg/tick)

FEEDING TIME (DAY)

FIG. 2

SUBSTITUTE SHEET (RULE 26)
FIG. 9

MW (kDa)

78 72 70 66 58.5 55

H₈ H₆ H₄ H₂ H₀ → TOP

24 23.5 22.9 21.5 15 → BOTTOM

FIG. 10

MW (kDa)

A B C D E F G H

180 →
116.5 →
84 →
56 →
48.5 →
36.5 →
26.5 →
**FIG. 19**

LAST WASHING FRACTION AGAROSE IGFBP IN HAEM.

MW kDa

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<th>pH 2.6</th>
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<td>90</td>
<td>96</td>
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MW kDa

~87 ~98 ~78

66

45

36

29

24

20

14

**FIG. 21**

ANTIGEN IN BLOOD:
TOTAL PROTEIN OF SGED 
(15 µg PER LANE)

MW kDa

G: PIG 523 524 525 526

29 IGFBPMA

23-25 MB

19-21 MC

SUBSTITUTE SHEET (RULE 26)
IGBP-MC:

ATGATGTCCTTTGC CCTCGC GTGTTTGCCTTGC ATGCTGTCATGC

MMSFAVSAVLALAFAVNVAHG

GGCTGACAATACCCACGATGCTAAATCAAGCCAAACACTAGAGCTGACTTCCACG

AVOCYHOLIKCSONYSAREIMG

GTGGTAACTAGGGATCCTGCTGCTGGCCACCCAGATGACTGCGCAAGATCAGTTTGGT

VVTRDADVGHQMRTAKIKLRV

TA CGACACCATCTTGAAGAAGAACGAGATGAGAGTCGACTCTTCAAAGCTCAAAGCACA

YD TILKELKMRVALFTPQGT

GTGCTACCATGCTATGTGAGGTTTCGATCGCTTGCTATGTATGGTTGGAAGATGTACCA

VVPCIEGFGSCVYDVCDVP

GAGAAACAGTCATGGACAGCGACCAAGTGGGTCCTGGGAAGCGACAGCGACAGTG

ENKVTMWTTKCPVPKPGTYWR

AACCTGGTGGTTCGCACGAGATGGTGAGCCGATGAGGCGGTCGACAGCTTTATC

NLFKVRVSPRMKLKHIGNGNL

GCTGCTCTTGTGTTGGAAGAAGCGGTAGAACCTTTGCTGCGCAAGGACTCCACCTCGGA

AALMLESKKGKLSCQALHLR

GTCTTCAACAGCGCCGGCCAGACCAGCTCAGTTTGGATTAGTGCTGCCACATGCATGACGATG

VFKQRPTTDIWD*

AAACAGGGCCCATGAAAGACATCGACTTGGTGCGAATACGAGACTGATGG

601

FIG. 27C
**FIG. 31**

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**FIG. 32**

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**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

| IPC 6 | C12N15/12 | C07K14/435 | C07K16/18 | A61K39/00 |

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

| IPC 6 | C07K |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>A</td>
<td>WO-A-94 02169 (PITMAN MOORE INC; SMITH WILLIAM DAVID (GB); SMITH STUART KEVIN (GB) 3 February 1994</td>
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<td>P, X</td>
<td>PARASITOLOGY, vol. 109, no. 4, November 1994 pages 525-530, WANG ET AL 'EXCRETION OF HOST IMMUNOGLOBULIN IN TICK SALIVA AND DETECTION OF IGG-BINDING PROTEINS IN TICK HAEMOLYMPH AND SALIVARY GLANDS' see the whole document</td>
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Further documents are listed in the continuation of box C.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Area

| Patent family members are listed in annex. |

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<th>Date of the actual completion of the international search</th>
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<td>14 July 1995</td>
<td>07.06.95</td>
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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016

Authorized officer

Sitch, W
### INTERNATIONAL SEARCH REPORT

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.:
   - because they relate to subject matter not required to be searched by this Authority, namely:
     - Remark: Although claims 14(completely) and 18–21(partially) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition

2. **☐** Claims Nos.:
   - because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **☐** Claims Nos.:
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. **☐** As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. **☐** As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. **☐** As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. **☐** No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- **☐** The additional search fees were accompanied by the applicant's protest.
- **☐** No protest accompanied the payment of additional search fees.
<table>
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<td>WO-A-9402169</td>
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