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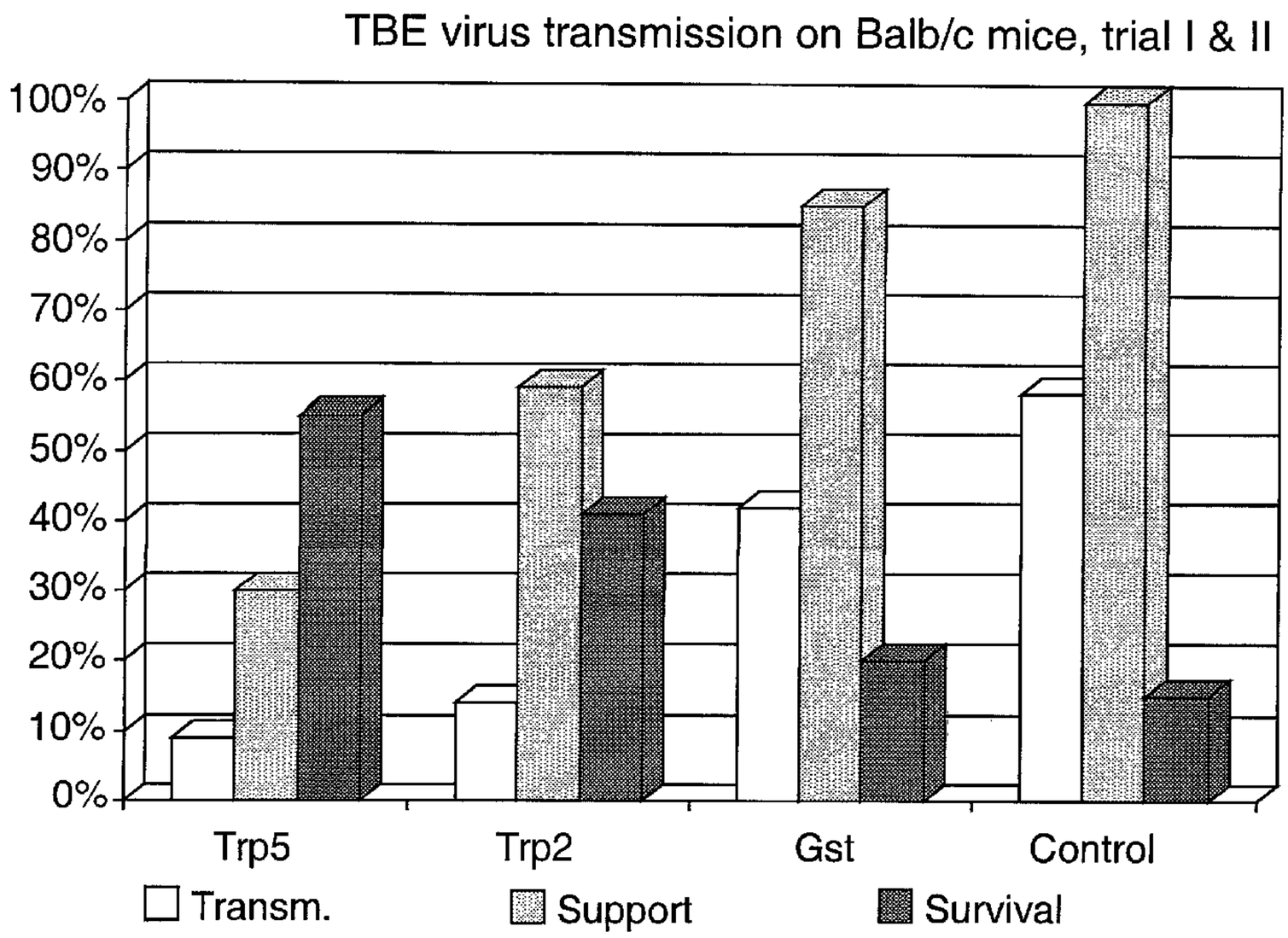

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VACCINES AGAINST ARTHROPOD-BORNE INFECTIOUS DISEASES

The present invention relates to the use of a protein termed 64p in the production of vaccines for protecting animals against the bite of blood-sucking ectoparasites and against the transmission of viruses, bacteria and other pathogens by such ectoparasites.

Blood-sucking ectoparasites, such as mosquitoes, horseflies, tsetse flies, fleas, lice, mites and ticks, are extremely effective as transmitters of disease. Mosquito borne diseases include Malaria (*Plasmodium* parasites transmitted by *Anopheles* mosquitoes), Dengue Fever, Yellow Fever and Arboviral Encephalitides (such as Eastern Equine Encephalitis, Japanese Encephalitis, La Crosse Encephalitis, St. Louis Encephalitis (*Culex pipiens* mosquitoes), Western Equine Encephalitis and West Nile Virus Encephalitis), Lymphatic filariasis (elephantiasis). Other diseases that are borne by blood-sucking ectoparasite vectors include plague (flea); Schistosomiasis (flatworms); trypanosomiasis (tsetse fly), Leishmaniasis (sandfly), and Onchocerciasis (blackfly).

Taking the tick as an example, these arthropods are able to transmit protozoan, rickttsial and viral diseases of livestock, which are of great economic importance world-wide. Losses to the livestock industry, in particular the production of cattle and small ruminants in tropical and sub-tropical areas, have been estimated to be in the range of several billion US dollars annually. In many developing countries, tick-borne protozoan diseases, including *Theileria parva* which causes the usually fatal East Coast Fever (Norval *et al.*, 1992a; Norval *et al.*, 1992b), babesioses and rickettsial diseases such as anaplasmoses, cowdriosis and tick-associated dermatophilosis, are major health and management problems of livestock. Furthermore, tick pests also cause considerable damage to animals' skin, thereby affecting the leather industry. Ticks also act as transmitters of human disease, including Lyme disease (*Borrelia burgdoreferi*, transmitted by *Ixodes scapularis*), Southern Tick-Associated Rash Illness (STARI), Babesiosis, Ehrlichiosis, Rocky Mountain Spotted Fever (caused by *Rickettsia rickettsia* transmitted by *Dermacentor variabilis*) and Crimean-Congo Haemorrhagic Fever.

Normally, these disease agents can only be transmitted by the bite of an infected ectoparasite. For example, ticks normally become infected by taking a blood meal from an infected animal. Male, female and immature (nymphs and larvae) ticks feed on blood and all stages are capable of transmitting disease agents. There are four stages in the life cycle of a tick: egg, larva, nymph, and adult. It generally takes several months to two years to complete this life cycle. A blood meal is taken in all except the egg stage. After each blood meal, the cuticle is shed and the tick matures to its next life stage. Thus it is possible for a tick to transmit disease organisms three times in its life. It is also possible to become infected by handling infected ticks, such as when removing ticks from a pet, when infective tick body fluids are introduced into a wound or mucus membrane.

In an effort to combat tick-transmitted diseases, a number of attempts have been made to immunize animals against ticks using extracts of whole ticks or of tick gut. Certain reports have used recombinant tick proteins (see, for example, International patent application WO88/03929). WO01/80881 reports the generation of vaccines that incorporate a protein termed 64P and fragments thereof. However, despite such developments, the only commercially-available tick vaccines are active only against the adult stage of a few tick species and show variation in efficacy depending on the geographical location of the species. No vaccines have yet been developed that provide resistance across entire populations of vaccinated animals or against parasites at every stage of their life cycle.

There is a great need to reduce the incidence of infectious diseases that are caused by blood-feeding ectoparasites, particularly in tropical and sub-tropical regions, where such ectoparasites, and the disease agents carried by them, are endemic. A large number of strategies are being pursued in order to try and eradicate these diseases, and these mainly focus on attempting to limit the numbers of the ectoparasites themselves. However, to date, none of these strategies has yet shown any enduring success. Indeed, as the global climate warms, it is likely that areas not previously afflicted by infectious diseases such as malaria will become vulnerable. The recent outbreak of human encephalitis cases in the north-eastern United States, caused by mosquito-borne West Nile virus, serves to illustrate the potential problems facing areas that are currently temperate over the coming years.

There therefore exists a great need for an effective vaccine to combat diseases that are transmitted by blood-feeding ectoparasites. It has now been discovered that a protein termed 64p, originally isolated in the tick, is useful as a vaccine component.

Summary of the invention

According to one aspect, the present invention relates to the use of an immunogenic 64p protein consisting of the amino acid sequence as set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, or 14, an immunogenic fragment thereof or an immunogenic homologue of said 64p protein or fragment having at least 50% identity thereto, or a plasmid DNA vector encoding said protein, fragment or homologue; for the preparation of a vaccine composition for the prevention of tick-borne encephalitis (TBE) and/or to reduce the transmission of the TBE virus to uninfected ticks; wherein said vaccine composition is for administration to animals.

According to another aspect, the present invention relates to a vaccine composition comprising immunogenic 64p protein consisting of the amino acid sequence as set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, or 14, an immunogenic fragment thereof or an immunogenic homologue of said 64p protein or fragment having at least 50% identity thereto, or a plasmid DNA vector encoding said protein, fragment or homologue; for use in the prevention of tick-borne encephalitis (TBE) and/or reduction in transmission of the TBE virus to uninfected ticks; wherein said vaccine composition is for administration to animals.

According to the present invention, there is provided a vaccine effective against the transmission of an infectious disease borne by an ectoparasite, said vaccine comprising as

an active component a 64p protein consisting of the sequence presented in Figure 1, a fragment thereof or a homologue of said 64p protein or protein fragment that exhibits at least 50% sequence identity with said protein or protein fragment.

Immunisation of an animal with such a vaccine is shown herein to cause the generation of antibodies that are effective against a wide variety of ectoparasite species. The vaccine is also shown to impart protection against the transmission of an infectious disease via a blood-sucking ectoparasite.

Although the Applicant does not want to be confined to any particular theory, it appears that the vaccines of the invention work in the following way. Immunisation of a host species with the 64p protein, fragment or homologue elicits an immune response against the ectoparasite protein. This stimulates an inflammatory response that boosts the immune status of vaccinated animals. However, additionally, the 64p protein and fragments thereof have been discovered to contain epitopes that also exist in proteins that are present in the salivary glands, gut and haemolymph of a large number of ectoparasite species. This cross-reactivity makes the vaccines of the invention particularly advantageous, since ingestion of blood, and thus host antibodies, into the ectoparasite guarantees delivery of the active agent to the parasite. In this manner, the vaccines of the invention target species that feed transitorily, such as mosquitoes and horseflies, as efficiently as those species that remain attached to their host for a significant period of time, such as ticks.

The nature of the immune response that the vaccines of the invention impart is responsible for the decreased transmission of the agent that causes the infectious disease borne by the ectoparasite. Because of the presence in the host of antibodies that recognise not only a protein species present in the ectoparasite saliva, but also a protein species that is found in the gut, the blood-feeding event, however transitory, is sufficient to allow the transmission to the ectoparasite of antibodies that lead to its death. Immunisation of host animals with a vaccine according to the invention thus leads to a decrease in the actual numbers of ectoparasites, as well as a concomitant decrease in the numbers of ectoparasites carrying disease-causing agents. This has a significant effect on the incidence of disease *per se*.

A large number of ectoparasite species exist in various parts of the world, although their incidence tends to be concentrated in tropical and sub-tropical regions, where they, and diseases carried by them, are endemic. These species vary greatly in type and adopt widely differing feeding strategies, ranging from transient feeders such as mosquitoes, horseflies,

sandflies, blackflies, tsetse flies, fleas, lice and mites, down to flatworms and ticks, some of which may feed for long periods of time. All of these ectoparasite species are suitable targets for the vaccines of the invention.

What is common between all these ectoparasite species is that they ingest either blood, lymph or they feed on host skin products, meaning that any antibodies present in their host are automatically internalised into the ectoparasite. This provides an advantageous and automatic route of administration for antibody and, provided that the antibody is reactive against an ectoparasite protein, means that a well-organized immunisation regime can result in the complete eradication of the parasite within the area concerned. Ectoparasites that feed on blood are particularly preferred targets for the vaccines of the invention.

The vaccines of the invention are particularly efficacious against tick species. Examples of such targeted tick species are *Rhipicephalus appendiculatus, R. sanguineus, R. bursa, Amblyomma variegatum, A. americanum, A. cajennense, A. hebraeum, Boophilus microplus, B. annulatus, B. decoloratus, Dermacentor reticulatus, D. andersoni, D. marginatus, D. variabilis, Haemaphysalis inermis, Ha. leachii, Ha. punctata, Hyalomma anatolicum anatolicum, Hy. dromedarii, Hy. marginatum marginatum, Ixodes ricinus, I. persulcatus, I. scapularis, I. hexagonus, Argas persicus, A. reflexus, Ornithodoros erraticus, O. moubata moubata, O. m. porcinus, and O. savignyi.*

The vaccines of the invention are also particularly efficacious against mosquito species. Examples of targeted mosquito species are those of the *Culex*, *Anopheles* and *Aedes* genera, particularly *Culex quinquefasciatus, Aedes aegypti* and *Anopheles gambiae*.

The vaccines of the invention are also particularly efficacious against flea species, such as *Ctenocephalides felis* (the cat flea).

As discussed above, blood-sucking ectoparasites are extremely effective as transmitters of infectious disease. Examples of disease agents, the transmission of which may be prevented or reduced using a vaccine according to the present invention include those transmitted by mosquitoes, horseflies, sandflies, blackflies, tsetse flies, fleas, lice, mites, flatworms and ticks. Examples of mosquito-borne diseases include Malaria, Dengue Fever, Yellow Fever and Arboviral Encephalitides, including Eastern Equine Encephalitis, Japanese Encephalitis, La Crosse Encephalitis, St. Louis Encephalitis, Western Equine Encephalitis and West Nile Virus Encephalitis and Lymphatic filariasis. Other diseases whose transmission may be prevented include plague (flea); schistosomiasis (flatworms); trypanosomiasis (tsetse fly),

Leishmaniasis (sandfly), and Onchocerciasis (blackfly). Examples of tick-transmitted diseases whose transmission may be blocked include protozoan, rickettsial and viral diseases of livestock, including East Coast Fever, babesioses, anaplasmoses, cowdriosis and tick-associated dermatophilosis, and human diseases, such as Lyme's Disease, Southern Tick-Associated Rash Illness (STARI), Babesiosis, Ehrlichiosis, Rocky Mountain Spotted Fever, tularemia, tick-borne relapsing fever, tick-borne encephalitis (TBE) and Crimean-Congo Haemorrhagic Fever. The particular utility of the vaccines of the invention in preventing transmission of TBE virus has been illustrated herein.

Preferably, the vaccines of the invention are effective against transmission of human diseases. However, these vaccines are also effective against the transmission of diseases in mammals (particularly livestock), birds, reptiles and fish.

By "64p" protein is meant a protein comprising the sequence presented in Figure 1 herein, or a homologue thereof. This protein and its properties are described in detail in co-owned, co-pending International patent application WO01/80881, the content of which is incorporated herein in its entirety. This protein contains at least one immunogenic epitope that is present in all blood-feeding ectoparasites that have been tested so far. It thus appears as if one single vaccine composition may be effective as a broad spectrum vaccine against all of the ectoparasite species that produce a protein containing this common epitope. The availability of a single vaccine that is effective against a number of different ectoparasite species will reduce the cost of administering the vaccine and will thus be advantageous over currently available vaccines.

The sequence in Figure 1 is the 64p sequence from the tick *Rhipicephalus appendiculatus*. This protein possesses a sequence typical of a structural protein, and appears to be secreted in the saliva of ticks. The sequence comprising the first 40 amino acids of the cement protein is strongly collagen-like, whereas the rest of the sequence resembles keratin. Homology searches conducted with the sequence of this protein reveals that the highest level of homology for all searched sequences in the Genbank database (http://www.ncbi.nlm.nih.gov) was 51%, for mouse epidermal keratin subunit I. The protein is glycine-rich and contains several repeats of the motif (C/S) 1-4 (Y/F), resembling structural proteins from *Drosophila melanogaster* (cuticular protein) and other insect egg shells, as well as vertebrate cytokeratins including mammalian keratin complex 2 basic protein, mouse keratin, human keratin, collagen type IV alpha, and IPIB2 precursor.

The term "homologue" is meant to include reference to paralogues and orthologues of the 64p sequence explicitly identified herein, including, for example, the 64p protein sequence from other tick species, including *R. sanguineus, R. bursa, Amblyomma variegatum, A. americanum, A. cajennense, A. hebraeum, Boophilus microplus, B. annulatus, B. decoloratus, Dermacentor reticulatus, D. andersoni, D. marginatus, D. variabilis, Haemaphysalis inermis, Ha. leachii, Ha. punctata, Hyalomma anatolicum anatolicum, Hy. dromedarii, Hy. marginatum marginatum, Ixodes ricinus, I. persulcatus, I. scapularis, I. hexagonus, Argas persicus, A. reflexus, Ornithodoros erraticus, O. moubata moubata, O. m. porcinus, and O. savignyi.* The term "homologue" is also meant to include the 64p protein sequence from mosquito species, including those of the *Culex*, *Anopheles* and *Aedes* genera, particularly *Culex quinquefasciatus, Aedes aegypti* and *Anopheles gambiae*; flea species, such as *Ctenocephalides felis* (the cat flea); horseflies; sandflies; blackflies; tsetse flies; fleas; lice; mites; leeches; and flatworms.

Methods for the identification of homologues of the 64p protein will be clear to those of skill in the art. For example, homologues may be identified by homology searching of sequence databases, both public and private. Conveniently, publicly available databases may be used, although private or commercially-available databases will be equally useful, particularly if they contain data not represented in the public databases. Primary databases are the sites of primary nucleotide or amino acid sequence data deposit and may be publicly or commercially available. Examples of publicly-available primary databases include the GenBank database (http://www.ncbi.nlm.nih.gov/), the EMBL database (http://www.ebi.ac.uk/), the DDBJ database (http://www.ddbj.nig.ac.jp/), the SWISS-PROT protein database (http://expasy.hcuge.ch/), PIR (http://pir.georgetown.edu/), TrEMBL (http://www.ebi.ac.uk/), the TIGR databases (see http://www.tigr.org/tdb/index.html), the NRL-3D database (http://www.nbrfa.georgetown.edu), the Protein Data Base (http://www.rcsb.org/pdb), the NRDB database (ftp://ncbi.nlm.nih.gov/pub/nrdb/README), the OWL database (http://www.biochem.ucl.ac.uk/bsm/dbbrowser/OWL/) and the secondary databases PROSITE (http://expasy.hcuge.ch/sprot/prosite.html), PRINTS (http://iupab.leeds.ac.uk/bmb5dp/prints.html), Profiles (http://ulrec3.unil.ch/software/PFSCAN_form.html), Pfam (http://www.sanger.ac.uk/software/pfam), Identify (http://dna.stanford.edu/identify/) and Blocks (http://www.blocks.fhcrc.org) databases. Examples of commercially-available

databases or private databases include PathoGenome (Genome Therapeutics Inc.) and PathoSeq (Incyte Pharmaceuticals Inc.).

Typically, greater than 30% identity between two polypeptides (preferably, over a specified region) is considered to be an indication of functional equivalence and thus an indication that two proteins are homologous. Preferably, proteins that are homologous to the 64p protein have a degree of sequence identity with the 64p protein, or with active fragments thereof, of greater than 55%. More preferred homologues have degrees of identity of greater than 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 99%, respectively with the 64p protein, or with active fragments thereof. Percentage identity, as referred to herein, is as determined using BLAST version 2.1.3 using the default parameters specified by the NCBI (the National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) [Blosum 62 matrix; gap open penalty=11 and gap extension penalty=1].

Homologues of the 64p protein include mutants containing amino acid substitutions, insertions or deletions from the wild type sequence, provided that the immunogenicity of the wild type protein sequence is retained. Mutants thus include proteins containing conservative amino acid substitutions that do not affect the function or activity of the protein in an adverse manner. This term is also intended to include natural biological variants (e.g. allelic variants or geographical variations within the species from which the tissue cement proteins are derived). Mutants with improved immunogenicity from that of the wild type protein sequence may also be designed through the systematic or directed mutation of specific residues in the protein sequence.

For the avoidance of doubt, also embraced by the term "homologues" are those proteins whose encoding genes have not yet been currently cloned, but which are cloned in the future. Methods for cloning genes that are homologues of the proteins of the invention will be known to those of skill in the art. For example, a nucleic acid molecule from the gene encoding 64p from *R. appendiculatus* (see Figure 1) as described above may be used as a hybridization probe for RNA, cDNA or genomic DNA isolated from an ectoparasite, in order to isolate full-length cDNAs and genomic clones encoding the equivalent 64p protein in this species. In this regard, the following techniques, among others known in the art, may be utilised and are discussed below for purposes of illustration.

One such method is to probe a genomic or cDNA library with a natural or artificially-designed probe using standard procedures that are recognised in the art (see, for example, "Current Protocols in Molecular Biology", Ausubel *et al.* (eds). Greene Publishing Association and John Wiley Interscience, New York, 1989,1992). Probes comprising at least 15, preferably at least 30, and more preferably at least 50, contiguous bases that correspond to, or are complementary to, nucleic acid sequences from an appropriate encoding gene, such as that set out in Figure 1 herein.

Such probes may be labelled with an analytically-detectable reagent to facilitate their identification. Useful reagents include, but are not limited to, radioisotopes, fluorescent dyes and enzymes that are capable of catalysing the formation of a detectable product. Using these probes, the ordinarily skilled artisan will be capable of isolating complementary copies of genomic DNA, cDNA or RNA polynucleotides encoding proteins of interest from human, mammalian or other animal sources and screening such sources for related sequences, for example, for additional members of the family, type and/or subtype.

In many cases, isolated cDNA sequences will be incomplete, in that the region encoding the polypeptide will be cut short, normally at the 5' end. Several methods are available to obtain full length cDNAs, or to extend short cDNAs. Such sequences may be extended utilising a partial nucleotide sequence and employing various methods known in the art to detect upstream sequences such as promoters and regulatory elements. For example, one method which may be employed is based on the method of Rapid Amplification of cDNA Ends (RACE; see, for example, Frohman *et al.*, Proc. Natl. Acad. Sci. USA (1988) 85: 8998-9002). Recent modifications of this technique, exemplified by the Marathon™ technology (Clontech Laboratories Inc.), for example, have significantly simplified the search for longer cDNAs. A slightly different technique, termed "restriction-site" PCR, uses universal primers to retrieve unknown nucleic acid sequence adjacent a known locus (Sarkar, G. (1993) PCR Methods Applic. 2:318-322). Inverse PCR may also be used to amplify or to extend sequences using divergent primers based on a known region (Triglia, T., *et al.* (1988) Nucleic Acids Res. 16:8186). Another method which may be used is capture PCR which involves PCR amplification of DNA fragments adjacent a known sequence in human and yeast artificial chromosome DNA (Lagerstrom, M. *et al.* (1991) PCR Methods Applic. 1: 111-119). Another method which may be used to retrieve unknown sequences is that of Parker, J.D. *et al.* (1991); Nucleic Acids Res. 19:3055-3060).

Additionally, one may use PCR, nested primers, and PromoterFinder™ libraries to walk genomic DNA (Clontech, Palo Alto, CA). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Also, random-primed libraries are preferable, in that they will contain more sequences that contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into 5' non-transcribed regulatory regions.

Methods for DNA sequencing and analysis are of course well known and are generally available in the art. Preferably, the sequencing process may be automated using machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno, NV), the Peltier Thermal Cycler (PTC200; MJ Research, Watertown, MA) and the ABI Catalyst and 373 and 377 DNA Sequencers (Perkin Elmer).

Fragments of the 64p protein are also useful as components of the vaccines of the present invention. Included as such fragments are not only fragments of the *Rhipicephalus appendiculatus* 64p protein that is explicitly identified herein in Figure 1, but also fragments of homologues of this protein. Such homologous fragments will typically possess greater than 30% identity with the *R. appendiculatus* 64p sequence, although more preferred homologues will display degrees of identity of greater than 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 99%, respectively with the 64p protein fragments that are explicitly identified herein.

For example, short stretches of peptide derived from immunogenic portions of 64p proteins may be particularly useful as immunogens. Such short stretches of polypeptide sequence are simple to produce in large quantities, either synthetically or through recombinant means. Protein fragments may in many instances be preferred for use in the vaccines of the invention, since these fragments are likely to fold into conformations not adopted by the full length wild type 64p sequence. Since some 64p proteins are likely to have evolved so as to resemble the tissues of the host skin and thus to avoid provoking a host immune response against the tick, such unnatural forms of tick cement proteins that expose 'hidden' epitopes are likely to be of particular use in the vaccines of the present invention.

Examples of fragments of 64p proteins that are useful for inclusion in the vaccine compositions of the invention include various fragments that have been generated recombinantly by the inventors (see WO01/80881), and homologues of these fragments and mutants of the kind discussed above. As will be apparent to the skilled reader, similar fragments to those that are explicitly disclosed herein may be prepared from ectoparasite species other than ticks.

The details of the *R. appendiculatus* fragments described herein are as follows.

The fragment termed 64trp1 (SEQ ID NO: 17) is a small soluble C-terminal fragment of the 64P protein consisting of 29 amino acids (residues 103-132 inclusive of the sequence of Figure 1) cloned as a glutathione-S-transferase (GST)/histidine tag fusion protein with a molecular weight of around 30 kDa.

The fragment termed 64trp2 (SEQ ID NO: 18) refers to a small soluble N-terminal fragment of the 64P protein consisting of 51 amino acids (residues 1-51 inclusive of the sequence of Figure 1) cloned as a glutathione-S-transferase (GST)/histidine tag fusion protein with a molecular weight of around 33 kDa.

The fragment termed 64trp3 (SEQ ID NO: 19) refers to a larger soluble N-terminal fragment of 64P protein consisting of 70 amino acids (residues 1-70 inclusive of the sequence of Figure 1) cloned as a glutathione-S-transferase (GST)/histidine tag fusion protein with a molecular weight of around 36 kDa.

The fragment termed 64trp4 (SEQ ID NO: 20) is a soluble C-terminal fragment of 64P protein consisting of 63 amino acids (residues 69-132 inclusive of the sequence of Figure 1) cloned as a glutathione-S- transferase (GST) /histidine tag fusion protein with a molecular weight of around 35 kDa.

The fragment termed 64trp5 (SEQ ID NO: 21) is the full-length clone of 64P protein sequence consisting of 133 amino acids cloned as a GST fusion protein (i. e. minus the histidine tag). This protein is soluble and has a molecular weight of 41 kDa.

The fragment termed 64trp6 (SEQ ID NO: 21 + a His Tag) refers to the full-length clone of 64P protein consisting of 133 amino acids cloned as a glutathione-S-

transferase (GST) /histidine tag fusion protein. This fragment is insoluble and has an approximate molecular weight of around 42 kDa.

These protein fragments, and homologues thereof, are particularly preferred components for incorporation in the vaccines of the invention. These fragments may be expressed as soluble protein, or may alternatively be expressed in inclusion bodies and purified under denaturing

conditions. For example, the construct 64trp6 as isolated from *R. appendiculatus* has been prepared as a denatured protein expressed in inclusion bodies and demonstrated to be immunogenic in this form.

Immunisation with these protein fragments, followed by attachment of ectoparasite, results in inflammation at the attachment site and subsequent death of the ectoparasite. The skilled reader will appreciate that the presence of the heterologous GST and HIS tag sequences is purely for convenience of protein production. These stretches of sequence are not considered to be essential to this aspect of the invention.

Conveniently, the vaccines according to the invention contain a 64p protein, fragment thereof or homologue thereof, expressed in recombinant form. Recombinantly-expressed protein is inexpensive to produce and, using the now standard techniques of genetic engineering, allows the simple manipulation of gene sequences to give a desired protein product.

It is preferred that the vaccines of the invention are effective against both adult and immature forms of the ectoparasite. The term "immature" is meant to include both nymph and larval forms of the ectoparasite. This means that the whole ectoparasite population may be targeted using the vaccine, so increasing the efficiency of eradication of ectoparasite and infectious disease causing agent.

The vaccines may specifically target adult or immature forms of ectoparasites, but will preferably target all parasitic stages of the life cycle. Of the fragments specifically exemplified herein, 64trp2-, 64trp3-, 64trp5- and 64trp6-immunised animals has been found to cause significant mortality in tick nymphs or adult ticks or both nymphs and adults, depending on the tick species, and these fragments are thus particularly preferred.

According to a further embodiment of the invention, there is provided a cocktail vaccine comprising, in addition to the 64p protein, fragment or homologue, a second active agent. The second active agent may preferably be a second immunogenic protein, or protein fragment derived from a blood-feeding ectoparasite. More preferably, the second immunogenic protein, fragment or homologue is a 64p protein or protein fragment.

The second active agent may be a vaccine against an infectious disease. It has been found that whilst certain commercially available vaccines against infectious disease are effective in abrogating the effects on the host of the virus that causes the infectious disease and thus in protecting against lethal challenge with virus-infected ticks, these vaccines do not

protect the host against infection. This is shown herein (see Example 3.10) by the ability of immunised hosts to support virus transmission to uninfected ticks feeding upon them.

The cocktail vaccine produced by the combination of a 64p-based vaccine according to the invention and a vaccine against an infectious disease would be effective in preventing viral transmission through the properties of the 64p components described above, and would also protect against lethal viral challenge through the properties of the vaccine against infectious disease. Such a combined vaccine should thus protect against both host infection and death.

Preferably, the vaccine against an infectious disease used as the second agent is a vaccine against TBE. Examples of commercially-available TBE vaccines are known to those of skill in the art.

Optionally, the cocktail vaccine may contain an adjuvant.

For example, any two or more immunogenic 64p proteins, protein fragments or functional equivalents may be used as components of such a cocktail vaccine, and may be from different or from the same tick species. For example, it may be desired to generate a vaccine that specifically targets more than one ectoparasite, or that targets different proteins from the same ectoparasite. In this manner, it may be possible to generate a more efficacious vaccine with greater species coverage. Particularly preferred combinations of components include the combination of 64trp2, 64trp3, 64trp5 and 64trp6, the combination of 64trp2 and 64trp5, the combination of 64trp2 and 64trp6 and the combination of 64trp5 and 64trp6. These combinations are demonstrated herein to possess particular efficacy in targeting both adult and immature ticks, in conferring cross-species resistance and in blocking the transmission to the host of the disease-causing agent.

Vaccine compositions according to the invention may also comprise additional agents, for example, molecules that the ectoparasite uses to promote pathogen transmission, such as interferon regulators, complement inhibitors, chemokine regulators and immunoglobulin-binding proteins. In this way, other bioactive molecules that are released from the salivary glands of ectoparasites may be recognised as foreign by the host immune system and an immune response mounted.

A further aspect of the present invention comprises a vaccine containing a 64p protein, fragment or homologue fused to another molecule, such as a label, a toxin or other bioactive or immunogenic molecule. Particularly suitable candidates for fusion may be a molecule such

as glutathione-S-transferase or a histidine tag, although luciferase, green fluorescent protein or horse radish peroxidase may also be suitable. Linker molecules such as streptavidin or biotin may also be used, for example, to facilitate purification of the cement protein.

Fusion proteins may be created chemically, using methods such as chemical cross-linking. Such methods will be well known to those of skill in the art and may comprise, for example, cross-linking of the thiol groups of cysteine residues. Chemical cross-linking will in most instances be used to fuse tissue cement proteins to non-protein molecules, such as labels.

When it is desired to fuse a tissue cement protein to another protein molecule, the method of choice will generally be to fuse the molecules genetically. In order to generate a recombinant fusion protein, the genes or gene portions that encode the proteins or protein fragments of interest are engineered so as to form one contiguous gene arranged so that the codons of the two gene sequences are transcribed in frame.

Immunisation with naked, plasmid DNA encoding specific antigens has recently been acknowledged as an efficient method of presenting antigens to the mammalian immune system, resulting in strong humoral and cellular immune responses (Ulmer *et al.*, Science 1993, 259, 1745-1749). This technique, also referred to as DNA vaccination, has been successfully applied to generate antibodies directed against several proteins derived from viruses (Ulmer *et al.*, *loc cit.*; Cox *et al.*, J. Virol. 1993, 67, 5664-5667; Fynan *et al.*, Proc. Natl. Acad. Sci. USA 1993, 90, 11478-11482; Robinson *et al.*, Vaccine 1993, 11, 957-960; Wang *et al.*, 1993, DNA Cell Biol. 1993, 12, 799-805; Davis *et al.*, Hum. Mol. Genet. 1993, 2, 1847-1851; Xiang *et al.*, Virology 1994, 199, 132-140; Xiang *et al.*, Virology 1995, 209, 569-579; and Justewicz *et al.*, J. Virol. 1995, 69, 7712-7717), parasites (Sedegah *et al.*, Proc. Natl. Acad. Sci. USA 1994, 91, 9866-9870; Mor *et al.*, J. Immunol. 1995, 155, 2039-2046; and Yang *et al.*, Biochem. Bioph. Res. Comm. 1995, 212, 1029-1039) and bacteria (Anderson *et al.*, Infect. Immun. 1996, 64, 3168-3173), and, in several cases, a significant protective response has been elicited by the host. These DNA vaccines continuously stimulate the immune system, amplifying immunity and thereby reducing the cost of production and delivery as no booster injections are required.

Based on the available evidence, immunisation with plasmid DNA encoding the various 64p proteins is likely to be a useful technique to improve their anti-tick vaccine effects further. The method would involve direct injection of the host with a eukaryotic expression vector

such that one or more 64p proteins are expressed by *in vivo* transcription then translation of the corresponding sequence within the vaccinated host (humans, livestock, or other animals).

The vaccines of any one of the above-described aspects of the invention may additionally comprise an adjuvant. Suitable adjuvants to enhance the effectiveness of the immunogenic proteins according to the present invention include, but are not limited to, oil-in-water emulsion formulations (optionally including other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) those formulations described in PCT Publ. No. WO 90/14837. Other suitable adjuvants will be known to those of skill in the art and include Saponin adjuvants, such as TiterMax Gold (CytRx Corporation, 150 Technology Parkway, Atlanta Norcross, Georgia), Stimulon™ (Cambridge Bioscience, Worcester, MA), ISA Montanide 50, cytokines, such as interleukins, interferons, macrophage colony stimulating factor (M-CSF) or tumor necrosis factor (TNF).

According to a further embodiment of the invention, there is provided a monoclonal antibody that is reactive with a 64p protein and which is thus effective in blocking the transmission of a disease-causing agent. By "reactive" is meant that the antibody binds to one or more epitopes of a 64p protein with an affinity of at least 10^{-8}M, preferably at least 10^{-9}M, more preferably at least 10^{-10}M. According to a preferred embodiment of this aspect of the invention, the antibody or antiserum is reactive against analogues of the 64p protein, for example, 64p protein analogues from a number of different ectoparasite species, such as the tick, mosquito, sandfly, blackfly and so on. This aspect of the invention includes a method for the production of such an antibody or an antiserum, comprising immunising an animal with a 64p protein, fragment thereof, or homologue thereof as listed in any one of the above-described aspects of the invention.

According to a still further aspect of the invention, there is provided a process for the formulation of a vaccine composition comprising bringing a 64p protein, fragment or homologue into association with a pharmaceutically-acceptable carrier, optionally in conjunction with an adjuvant. The technology referred to as jet injection (see, for example, www.powderject.com) may also be useful in the formulation of vaccine compositions.

According to a still further aspect of the present invention, there is provided a method of immunising a mammal against an ectoparasite-transmitted disease or against a blood-feeding ectoparasite, comprising administering to an animal, a vaccine according to any one of the above-described aspects of the invention. Such an immunisation method may utilise

conventional means, but alternative methods of administering vaccines, such as through the use of jet injection may be equally effective or even preferable (see, for example, www.powderject.com; also Sarno *et al.* (2000) Pediatr. Infect. Dis. J. 19: 839-842).

The invention also provides a 64p protein, fragment thereof or homologue thereof, for use in a vaccine. The invention further provides for the use of a 64p protein, fragment thereof or homologue thereof as a component of a vaccine.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to the 64p protein from the tick, *Rhipicephalus appendiculatus*. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

Brief description of the Figures

Figure 1: Nucleotide and inferred amino acid sequences of 64p: Complete cDNA sequence and cDNA-inferred protein sequence of clone 64 (SEQ ID NO: 12). The putative signal sequence is given in bold. A possible glycosaminoglycan attachment site is underlined. The first 40 amino-acid piece of the mature protein is collagen-like, the remainder of the sequence resembles keratin. The protein is glycine-rich and contains several repeats of the motif (C/S)1-4(Y/F), which is also found in structural proteins from insect egg shells. The tyrosines may be involved in cross-linking by formation of dityrosine-bridges by phenoloxidases. * indicates the stop codon. Clone 21 is SEQ ID NO: 2, Clone 33 is SEQ ID NO: 4, Clone 24 is a SEQ ID NO: 8, Close 68 is SEQ ID NO: 10, Clone 64 is SEQ ID NO: 12, CemA is SEQ ID NO: 6 and Clone I is SEQ ID NO: 14.

Figure 2: Amino acid sequences of 64P protein fragments (64TRPs) expressed in *Escherichia coli*. P1/P2, P1/P3, P4/P5, P6/P5, P1/P5 and P7/P5 refer to primers used to subclone PCR products from 64P amino acid sequence into the plasmid pGEX-2T, for expression in *Escherichia coli* cells as truncated versions of 64P protein, *i.e.* 64trp2 (SEQ ID NO: 18, 51 amino acids), 64trp3 (SEQ ID NO: 19, 70 amino acids), 64trp1 (SEQ ID NO: 17, 29 amino acids), 64trp4 (SEQ ID NO: 20, 63 amino acids), 64trp5

(SEQ ID NO: 21, 133 amino acids without HIS.TAG) and 64trp6 (SEQ ID NO: 21 + a His Tag, 133 amino acids with HIS.TAG), respectively. Predicted possible cleavage signal peptide (amino acids 1 to 18) is underlined in green.

Figure 3: Histological studies of skin sections from hamsters immunised with different cocktails of 64trp proteins, post-feeding of *Ixodes ricinus* nymphs, stained with Haematoxylin and Eosin. (A) = histological section from skin of GST-immunised, control

hamster & (B), (C) & (D)= histological sections from skin of hamsters immunised with 64trp proteins, post-feeding with *I. Ricinus* nymphs, stained with haematoxylin & eosin stains; sections (A), (B) & (D) - magnification=20X; section (C) - magnification=40X; arrows: 1=epidermis, 2=dermis, 3 =subcutis & 4=cement cone; d=dentritic-like cells (i.e. Langerhan cells on cement cone), f=fibroblasts, l=lymphocytes & m=mast cells.

Figure 4: Immunoblots of different mosquito antigens probed with **(A)** anti-64trp2, **(B)** anti-64trp5, **(C)** anti-64trp6 and **(D)** anti-GST(control) sera, respectively; **(E)** Coomassie Blue stained 4-12% gradient gel showing the protein profile of the same mosquito antigens.

Lanes 2, 3, 4, 5 & 6 in all figures denote: **Lane 2**, salivary gland extract of *Anopheles gambiae*, **Lane 3** midgut extract of *Anopheles gambiae*, **Lane 4** midgut extract of *Aedes aegypti*, **Lane 5** midgut extract of *Culex quinquefasciatus* and **Lane 6** salivary gland extract of *Culex quinquefasciatus*, mosquitoes. Immunopositive bands labelled as **a - n**; faintly visible bands **l - n** refer to non-specific binding of proteins in the mosquito tissue extracts by the anti-GST control serum.

Lane **1** figures **A, B, C & D**: SeeBlue™ Plus2 protein molecular weight markers: 188 kD = Myosin, 98 kD = Phosphorylase B, 62 kD = Bovine serum albumin, 49 kD = Glutamic dehydrogenase, 38 kD = Alcohol dehydrogenase, 28 kD = Carbonic anhydrase, 17 kD = Myoglobin Red and 14 kD = Lysozyme; lane **1** figure **E**: Mark 12 protein markers: 200 kD = Myosin, 116.3 kD = *B*-galactosidase, 97.4 kD = Phosphorylase b, 66.3 kD = Bovine serum albumin, 55.4 kD = Glutamic dehydrogenase, 36.5 kD = Lactate dehydrogenase, 31 kD = Carbonic anhydrase, 21.5 kD = Trypsin inhibitor, 14.4 kD = Lysozyme and 6 kD = Aprotinin.

Figure 5: Cross-reactivity between *Ctenocephalides felis* (cat flea) antigens using antisera from guinea pigs immunised with recombinant R. appendiculatus 64trp proteins.

A, B, C, D & E immunoblots of whole flea extracts of *Ctenocephalides felis* fed on cats using GST **(A)**, 64trp2 **(B)**, 64trp3 **(C)**, 64trp5 **(D)** and 64trp6 **(E)** antisera, respectively; **F** Coomassie Blue stained 4-12% Bis-Tris gradient gel (NuPAGE-Novex) of the same extracts.

Lanes: **A/1, B/1, C/1, D/1 & E/1:** See Blue™ Plus 2 protein molecular weight markers (Novex): 98 kD=Phosphorylase b, 62 kD=BSA, 49 kD=Glutamine dehydrogenase, 38 kD=Alcohol dehydrogenase, 28 kD=Carbonic anhydrase, 17 kD=Myoglobin Red & 14

kD=Lysozyme. Lane: **F/1:** Mark 12™ protein moleculat weight markers 9Novex): 200 kD=Myosin, 116.3 kD=B-galactosidase, 97.4 kD=Phosphorylase b, 66.3 kD=BSA, 55.4 kD=Glutamic dehydrogenase, 36.5 kD=Lactate dehydrogenase, 31 kD=Carbonic anhydrase & 21.5 kD=Trypsin inhibitor.

Lanes: **B/2, C/2, D/2 & E/2**= *C. felis* whole extract of which immuno-positive bands were observed as **b**=110 kD, **c**=62 kD, **d**=75 kD, **e**=98 kD, **f**=75 kD, **g**=48-50 kD, **h**=28-31 kD, **i**=98-120 kD and **j**=50 kD, respectively. Lane: **A/2**= *C. felis* whole extract showing a faint immuno-positive band observed as **a**, due cross-reactivity of flea antigens with anti-GST antiserum.

Figures 6-8: Effect of 64TRP constructs in blocking TBE virus transmission in mice.

Examples

Expression of truncated cement proteins (64TRP) in bacteria is reported in WO01/80881.

This patent application also details the expression of the various 64TRP constructs, along with immunohistochemical studies using antiserum to 64TRP, and vaccination trials in Dunkin-Hartley Guinea pigs. It was also reported in this application that antisera raised against *Rhipicephalus appendiculatus* cement protein 64TRP were cross-reactive with antigenic epitopes in the salivary gland, midgut and haemolymph of adult female *R. appendiculatus* and with antigenic epitopes in the salivary glands, midgut and haemolymph of three other ixodid tick species. The results suggested that the candidate vaccine(s) derived from *R. appendiculatus* cement provide a broad spectrum vaccine that is effective against a number of different tick species.

On the basis of the observed cross-reactivities, 64trp6 of *R. appendiculatus* was selected as an immunogen for a vaccine trial. The results presented in this specification showed that putative vaccines derived against cement protein 64TRP of *R. appendiculatus* were cross-protective against adults and nymphs of *R. sanguineus*.

Following on from this work, additional work has now been performed that has revealed surprising findings relating to the cross-reactivity of 64TRP-based vaccines in insects, and the ability of these vaccines to confer resistance to infection.

Example 1: Cross-reactivity and Cross-protection Vaccine Trial with insects

1.1 Selection of immunogens

Candidate immunogens were identified on the basis of whether antiserum to the construct detected specific cross-reacting antigens in extracts of mosquitoes and fleas.

Cross-reactivity studies using immunoblotting with 64trp antisera showed detection of protein bands with mosquito extracts of *Anopheles gambiae* salivary gland and midgut, *Aedes aegypti* midgut, and *Culex quinquefasciatus* salivary gland and midgut (Figure 3).

Using 64trp2 antiserum (Figure 4A), two major bands (**a** and **b**) were detected in all extracts.

Antisera to 64 trp5 (Figure 4B) detected several less pronounced bands in midgut of *An. gambiae* and *C. quinquefasciatus*. Antiserum to 64trp6 detected a prominent band in *An. gambiae* midgut (Figure 4C).

The control antiserum raised against GST detected very faint bands in midgut of *An. gambiae* and *C. quinquefasciatus* that differed in size from those detected with 64trp antisera (Figure 4D).

Based on the availability of *A. aegypti* larvae, and the observed cross-reactivities, a vaccine trial was undertaken using 64trp2 and 5.

1.2 Treatments for vaccine trial:

- Group 1: Recombinant 64trp2 + 64 trp5 + Montanide ISA (2 mice)
- Group 2: Recombinant 64trp5 + Montanide ISA (2 mice)
- Group 3: Recombinant 64trp2 + Montanide ISA (2 mice)
- Group 4: GST (control) (2 mice)
- Group 5: Recombinant 64trp2 + 64 trp5 + Montanide ISA (2 guinea pigs)
- Group 6: Recombinant 64trp2 + Montanide ISA (2 guinea pigs)
- Group 7: Recombinant 64trp5 + Montanide ISA (2 guinea pigs)
- Group 8: GST (control) (2 guinea pigs)

Total Number of Animals = 8 mice + 8 guinea pigs

1.3 Route and dose:

Subcutaneous inoculation in the prescapular region either singly or as combined antigens into a single site.

Dose 50 µg antigen per guinea pig and 10 µg antigen per mouse.

1.4 Vaccination scheme:

1. Primer inoculation
2. First boost
3. Test bleed at 10 to 12 days post-inoculation
4. Second boost (if antibody titre <1/5000)
5. Test bleed at 10 to 12 days post-inoculation
6. Antibody titre >1/5000: challenge with *Aedes aegypti* mosquitoes.
7. Evaluate local inflammatory immune response to repeated mosquito feeding, and survival of fed mosquitoes.

1.5 Results

The results are summarised in Tables 1a and 1b. Overall, they show that putative vaccines derived against cement of *R. appendiculatus* were cross-reactive against *A. aegypti* mosquitoes.

(i) Feeding success

Adult female mosquitoes fed preferentially on young (5-6 week-old) mice compared with guinea pigs.

(ii) Inflammatory response

Local skin hypersensitivity reactions, observed as intense papular swellings, were observed on the abdomen and foot pads of mice by the third feeding.

(iii) Post-feeding mortality

Higher mortality was observed among mosquitoes exposed to 64trp-immunised animals compared to control animals.

(iv) Antibody titres

Antibody titres for Balb/c mice immunised with 64trp2 were > 1:64,000 and for C57/BI10 mice immunised with 64trp2, 1:64,000.

1.6 Conclusions

1. Antibodies raised against the 64trp proteins cross-react in immunoblots with antigenic epitopes in salivary gland and midguts of adult mosquitoes.

2. A host inflammatory response was observed in mice immunised with 64trp immunogens.

3. The mortality in mosquitoes fed on 64trp-immunised animals indicates that the tick cement protein is a candidate for developing anti-mosquito vaccines.

Table 1a. Effect of feeding *Aedes aegypti* mosquitoes on mice immunised with recombinant 64trp proteins

Mouse strain	64trp protein	Live mosquitoes		Dead mosquitoes		Total MM+FF	Mortality (%) FF
		MM	FF	MM	FF		
Balb/C	64trp2/5	19	9	0	5*	33	20.8
Balb/C	64trp5	14	13	0	8	35	30.1
Balb/C	64trp2	6	10	0	7*	23	41.2
Balb/C	GST (control)	19	16	0	0	35	0
C57/BI10	64trp2/5	10	15	1	6*	33	28.6
C57/BI10	64trp5	8	14	0	2	31	12.5
C57/BI10	64trp2	23	9	0	3	35	25
C57/BI10	GST (control)	22	7	0	1	30	12.5

Table 1b. Effect of feeding *Aedes aegypti* mosquitoes on guinea pigs immunised with recombinant 64trp proteins

Guinea pig no.	64trp protein	Live mosquitoes		Dead mosquitoes		Total MM+FF	Mortality (%) FF
		MM	FF	MM	FF		
1a	64trp2/5	19	21	0	8	48	27.6
1b	64trp2/5	26	19	1	6	52	24
2a	64trp2	19	14	0	2	35	12.5
2b	64trp2	14	18	0	9	41	33.3
3a	64trp5	16	21	1	13	51	38.2
3b	64trp5	16	11	0	6	33	35.3
4a	GST (control)	18	10	1	0	29	0
4b	GST (control)	19	9	0	1	26	10

*female mosquitoes observed dying during counting

Example 2: Antigenic cross-reactivity between *Rhipicephalus appendiculatus* and *Ctenocephalides felis* cat flea detected by immunoblotting using antisera to *R. appendiculatus* cement protein 64 trp constructs

Antisera to 64trp 2, 64trp5, and 64trp6 showed strong cross-reactivities in immunoblots of whole cat flea extract probed with the respective antisera (Figure 5). The results are summarised in Table 2 below. Single cross-reactions were also detected with anti-64trp3 and anti-GST sera. The cross-reactivities demonstrate the potential for developing an anti-flea vaccine using the tick cement protein.

Table 2. Cross-reactivity between *Rhipicephalus appendiculatus* and *Ctenocephalides felis* whole flea extract using sera from guinea pigs immunised with 64 trp recombinant antigens

Antiserum	Tick Antigens						
	R. appendiculatus						*C. felis*
	CC	SG	gut	H	N	L	
Anti-64trp2 ab' (50a.a. N-term. Frag. of 64P) Effective against RA Adult/nymph ticks (soluble antigen)	+	+	+	+	+	+	+
Anti-64trp3 ab' (70a.a. N-term. Frag. of 64P) Effective against RA Adult/nymphs ticks (soluble antigen)	+	+	+	+	+-	+	+
Anti-64trp5 ab' (133a.a. full- length clone of 64P) effective against RA nymph ticks (soluble antigen)	+	+	+	+	-	-	+
Anti-64trp6 ab' (133a.a. full- length clone of 64P) effective against RA nymph ticks (de-natured antigen)	+	+	+	+	+	+	+
Anti-GST ab' control antiserum	-	+	-	-	-	-	+

CC = tick cement cone extract; SG = salivary gland extract, gut = midgut extract; H = haemolymph; N = whole nymphal extract; L = whole larval extract; + = positive and - = negative reactions, respectively, to antisera used in immunoblots; ab' = antiserum

Example 3: Evaluation of 64p anti-tick vaccine constructs for their ability to protect mice against tick-borne encephalitis (TBE) virus infection.

3. 1 Selection of immunogens

Candidate immunogens were selected on the basis of whether antiserum to the 64TRP constructs detected specific cross-reacting antigens in extracts of *Ixodes ricinus*, the tick vector of TBE virus (see Table 3 of WO01/80881).

3.2 Treatments for vaccine trial (Trial 1):

- Group A: Recombinant 64trp2 + TiterMax Gold (TMG) (10 mice)
- Group B: Recombinant 64trp5 + TMG (10 mice)
- Group C: Recombinant 64trp2 + 64trp6 + TMG (10 mice)
- Group D: Recombinant 64trp5 + 64trp6 + TMG (10 mice)
- Group E: Recombinant 64trp2 + 64trp5 + TMG (10 mice)
- Group F: GST protein (10 mice)
- Group G: TMG (10 mice)
- Group H: untreated (10 mice)

Total number of animals = 80 Balb/c mice.

3.3 Route and dose:

Subcutaneous inoculation in the prescapular region either singly or as combined antigens into a single site. Final volume of inoculum = 200µl

Group A. 15µl TRP2 + 85µl PBS + 100µl TMG

Group B. 20µl TRP5 + 80µl PBS + 100µl TMG

Group C. 20µl TRP2 + 25µl TRP5 + 55µl PBS + 100µl TMG

Group D. 20µl TRP2 + 5µlTRP6 + 75µl PBS + 100µl TMG

Group E. 25µl TRP5 + 5µl TRP6 + 70µl PBS + 100µl TMG

Group F. 10µl GST + 90µl PBS + 100µl TMG

Group G. 100µl TM +100µl PBS Group H. untreated control group

3.4 Vaccination scheme:

All animals were immunized on the same day.

Serum samples were collected from the half of the exp. mice from sinus orbitalis in each of the 8 groups 12 to 14 days after immunisation (30µl to 50µl serum/animal) and then 25 days after immunisation from all remaining mice.

3.5 TBE virus infection and transmission

TBE virus donors used to infect the mice were field collected *Ixodes ricinus* female ticks (FIR) inoculated parenterally with TBE virus (Hypr strain), diluted 10^{-1}, volume 0.002 ml, in total 5000 PFU/tick. They were inoculated on either the same day or the day after immunisation of the mice.

Recipient ticks used to assay for virus transmission were uninfected *Ixodes ricinus* males (MIR) and nymphs (NIR) although male ticks were not tested. Mice were challenged with 1 infected FIR, 1 MIR, and 15 NIR all placed in one retaining chamber glued to each mouse.

No signs of inflammation were recorded during the trial.

The mice were infested with ticks either 32 days (mouse 1-5 in each group + C6 and C7) or 33 days (mouse 6-10 in each group minus C6 and C7) after immunisation. Each mouse was challenged with one infected *I. ricinus* female + one uninfected *I. ricinus* male and 15 *I. ricinus* nymphs. Ticks fed for 3 days and were then collected. Mice were observed for signs of illness/death for a 21 day period.

3.6 Lethal virus challenge

All surviving mice were inoculated intra-peritoneally with approximately 1000 plaque forming units of TBE virus (Hypr strain), 54 days after immunisation, and then monitored for 21 days.

Survivors were bled 20 days after lethal challenge.

3.7 Results (Trial 1)

The results are summarised in Table 3 and illustrated in Figure 6.

1. Highest % survival was shown by mice immunised with trp5 or trp2.
2. Transmission-blocking, as measured by the % tick infected, was particularly striking for trp5.
3. The percentage of mice that support virus transmission was lowest for trp5.

3.8 Trial II

Immunisation protocol - Antigens used

TRP-GST fusion proteins including: TRP2, TRP5, and GST protein (control);

Treatment groups

Two treatments:

1.TRP2 (**A**);

2.TRP5 (**B**);

Two controls:

1.GST protein (**C**);

2.unimmunised (**D**).

Mouse trial (Balb/c): 20 mice per treatment group

(**A**) 15ul TRP2 + 85ul PBS + 100ul TMG = 200ul final volume injected S.C.

(**B**) 20ul TRP5 + 80ul PBS + 100ul TMG = 200ul final volume injected S.C.

(**C**) 10ul GST + 90ul PBS + 100ul TMG = 200ul final volume injected S.C.

(**D**) unimmunised control group

(TMG=TiterMax Gold adjuvant)

Dates for pre-challenge procedures

1. Immunisations – single dose per mouse were performed on **7/11/01,** (i.e. no boostings were performed)

2. Serum samples – were collected third week post-immunisations (**27-28/11/01**) for serological assays (ELISAs) and mice were challenged at fourth week post-immunisations (**4-7/12/01**);

TBE virus infection via Ixodes ricinus infected adult female ticks co-feeding with uninfected I. r. nymphs was tested for transmission/survival studies (i.e. challenge)

Results:

The results for survival of mice after commencement of infected tick feeding (in days and date of death) and transmission rate of TBE virus are given below in Table 3:

Mouse no.	Survival in D (death date)	Ticks inf./fed (% infected)	Mouse no.	Survival in D (death date)	Ticks inf./fed (% infected)
A1	13 (13/12)	1/11 (9.1%)	B1	12 (12/12)	0/14 (0%)
A2	-	ND	B2	S; **10** (14/01)	0/3 (0%)
A3	10 (10/12)	0/8 (0%)	B3	**S**; S	0/10 (0%)
A4	18 (18/12)	0/8 (0%)	B4	11 (11/12)	5/12 (41.7%)
A5	**S**; S	0/10 (0%)	B5	S; **10** (14/01)	0/11 (0%)
A6	10 (10/12)	0/8 (0%)	B6	**S**; S	0/4 (0%)
A7	10 (10/12)	2/12 (16.7%)	B7	10 (10/12)	0/7 (0%)
A8	**S**; S	3/7 (42.9%)	B8	**S**; S	2/7 (28.6%)
A9	10 (10/12)	1/10 (10%)	B9	**S**; S	2/9 (22.2%)
A10	**S**; S	0/13 (0%)	B10	12 (12/12)	1/10 (10%)
A	**3/9 (33%)**	**7/87, 8.0%**	**B**	**6/10 (60%)**	**10/87, 11.5%**
C1	**S**; S	3/6 (50%)	D1	10 (10/12)	3/7 (42.8%)
C2	10 (10/12)	8/9 (88.9%)	D2	12 (12/12)	7/10 (70%)
C3	14 (14/12)	1/11 (9.1%)	D3	6 (06/12)	4/11 (36.4%)
C4	11 (11/12)	9/10 (90%)	D4	10 (10/12)	3/8 (37.5%)
C5	11 (11/12)	3/10 (30%)	D5	10 (10/12)	1/2 (50%)
C6	14 (14/12)	5/7 (71.4%)	D6	10 (10/12)	3/6 (50%)
C7	13 (13/12)	5/12 (41.7%)	D7	10 (10/12)	5/11 (45.5%)
C8	14 (14/12)	2/9 (22.2%)	D8	10 (10/12)	3/5 (60%)
C9	**S**; S	4/7 (57.1%)	D9	10 (10/12)	3/4 (75%)
C10	14 (14/12)	2/6 (33.3%)	D10	10 (10/12)	4/7 (57.1%)
C	**2/10 (20%)**	**42/87, 48.3%**	**D**	**0/10 (0%)**	**36/71, 50.7%**

Table 3

Notes:

ND: not done; **S** – surviving mouse; S – a mouse surviving 2nd challenge; NEG. – donor tick was not infected, a mouse and ticks feeding on that mouse excluded from the trial.

2nd challenge control mice – challenge Hypr ip 1000 PFU (04/01/2002):

1. D7, **2.** D8, **3.** D8, **4.** D8 – mean incubation period D**7.75**

A summary of TBE virus transmission in the BALB/c mice is given below in Table 4.

Mouse group & no.	**Nymphs infected/fed**	**% infected**
A1	1/11	9.1%
A2	ND	-
A3	0/8	0%
A4	0/8	0%
A5	0/10	0%
A6	0/8	0%
A7	2/12	16.7%
A8	3/7	42.9%
A9	1/10	10%
A10	0/13	0%
A	**7/87**	**8.0% (4/9)**
B1	0/14	0%
B2	0/3	0%
B3	0/10	0%
B4	5/12	41.7%
B5	0/11	0%
B6	0/4	0%
B7	0/7	0%
B8	2/7	28.6%
B9	2/9	22.2%
B10	1/10	10%
B	**10/87**	**11.5% (4/10)**
C1	3/6	50%
C2	8/9	88.9%
C3	1/11	9.1%
C4	9/10	90%
C5	3/10	30%
C6	5/7	71.4%
C7	5/12	41.7%
C8	2/9	22.2%
C9	4/7	57.1%
C10	2/6	33.3%
C=F	**42/87**	**48.3% (10/10)**
D1	3/7	42.8%
D2	7/10	70%
D3	4/11	36.4%
D4	3/8	37.5%
D5	1/2	50%
D6	3/6	50%
D7	5/11	45.4%
D8	3/5	60%
D9	3/4	75%
D10	4/7	57.1
D=H	**36/71**	**50.7% (10/10)**

Table 10

The combined results of TBE virus transmission on TRP immune Balb/c mice, for both Trial I & II are given below in Table 5.

Group of mice	Transmission	Support of transmis.	Survival
Group **A** (Trp2)	**14.3%** (22/154)	59% (10/17)	41.2% (7/17)
Group **B** (Trp5)	**9.4%** (16/180)	30% (6/20)	55% (11/20)
Group **C** = F (Gst)	**41.9%** (67/160)	85% (17/20)	20% (4/20)
Group **D** = H	**58.4%** (94/161)	100% (20/20)	15% (3/20)

Table 5

The results of Trial II are illustrated in Figure 7.

The combined results of Trials I and II are illustrated in Figure 8.

3.9 Conclusions

Protection against TBEV challenges (i.e. survival of mice) was observed in the TRP2 - and TRP5 -immunised groups (41% and 55%, respectively) compared with much lower survival rate in control groups of unimmunised and GST- immunized mice (15 and 20%, respectively). The infection rate in *I. ricinus* nymphs co-fed on mice immunised with 64TRP5 and 64TRP2 was greatly reduced (14% and 9%, respectively) as compared to nymphs co-fed on unimmunised and GST- immunised control animals (58% and 42%, respectively). Virus propagation (i.e. replication in host cells) was evident in mice from both control groups while only 30% and 59% of 64TRP5 - and 64TRP2 – immunised mice, respectively, supported TBE virus transmission.

Immunisation of Balb/c mice with tick derived recombinant 64TRP2 and 64TRP5 proteins was thus shown to protect the mice against lethal challenge with tick-infected TBE virus, as well as having a virus transmission-blocking effect, suggesting that these proteins provide a considerable degree of protection against virus transmission and against virus-induced death.

3.10 Comparison between efficacy of 64TRP constructs and commercial TBE vaccine

Experiments were performed to evaluate the efficacy of a commercial TBE vaccine and 64TRP constructs according to the invention. The data are presented below in Table 6.

These data show that mice immunised with the commercial TBE virus vaccine are protected against lethal challenge with virus-infected ticks (although 2 mice succumbed) but are not protected against infection as shown by their ability to support virus transmission to uninfected ticks feeding upon them.

This result contrasts with the impressive results for 64TRP5 showing markedly reduced levels of infection (and equally impressive protection given that the immunogen is tick derived and unrelated to the virus).

It is possible that the deficiencies of the commercial TBE vaccine can be explained by the fact that development of the commercial TBE vaccine was based on challenging mice with virus inoculated by needle & syringe and NOT delivered by the natural route - an infected tick. It appears that that tick-borne delivery of the virus is able to 'protect' the virus from host immunity to a limited degree that occasionally allows the virus to 'breakthrough' and cause disease/death.

This result strongly suggests that the efficacy of the constructs according to the invention in preventing viral transmission might be combined with the advantages of a TBE virus vaccine in protecting against lethal viral challenge with virus-infected ticks. Such a combined vaccine should protect against both infection and death.

Group of mice (immunized with)	Nymphs infected/fed (%)	Mice supporting trans./ used (%)*	Mice survived/used (%)
A (TRP2)	22/144 (**15%**)	10/16 (**62%**)	6/16 (**38%**)
B (TRP5)	16/173 (**9%**)	6/19 (**32%**)	10/19 (**53%**)
F (GST+TMG)	67/153 (**44%**)	17/19 (**90%**)	3/19 (**16%**)
H (untreated)	94/161 (**58%**)	20/20 (**100%**)	3/20 (**15%**)
I (TBE vaccine)	38/180 (**21%**)	12/17 (**71%**)	15/17 (**88%**)

* animals supporting transmission/animals in the experiment; % of animals supporting transmission

TABLE 6: A summary of transmission efficiency of TBE virus on laboratory mice (+/- immunized with 64TRP, or a TBE virus vaccine) and their survival after an infected tick bite; trial I & II.

SEQUENCE LISTING

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<130> 57630-NP

<140> CA 2,463,085
<141> 2002-10-07

<150> PCT/GB02/04546
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<150> GB 0124137.1
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<150> GB 0206575.3
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gcactaagtc taggagttgg aggcttcccg tccggtgctt cgcttggcag ccttagtggc 180

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			20					25					30						
Ala	Leu	Ser	Leu	Gly	Val	Gly	Gly	Phe	Pro	Ser	Gly	Ala	Ser	Leu	Gly				
		35					40					45							
Ser	Leu	Ser	Gly	Val	Thr	Leu	Ser	Gly	Ala	Gly	Ser	Ser	Val	Ser	Gly				
	50					55					60								
Arg	Pro	Gly	Ser	Pro	Gly	Ser	Ala	Gly	Pro	Ser	Ser	Gly	Pro	Ala	Val				
65					70					75					80				
Ser																			

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20 25 30

Gly Leu Gly Ala Ile Phe Gly Pro Gly Ala Tyr Pro Gly Phe Tyr Gly
35 40 45

Leu Asn Ser Val His Leu Leu Gly Gly Arg Phe His His Leu Phe Gly
50 55 60

Arg Phe Pro Pro Pro Pro Gly Ile Gly Ala Ala Glu Arg Arg Gly Thr
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Leu Ser Pro Tyr Pro Leu Asp Ile Asn Thr Val Gln Asp Pro Val Thr
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Gly His Pro Met Val Arg Val Val Tyr Gly Gly Val Leu Arg Glu Arg
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Pro Ile Pro Val Pro Gln Pro Tyr Pro Val Pro His Pro Arg Gln Val
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Pro Tyr Pro Val Pro Ser Pro Tyr Pro Val Pro Ile His Ser Asn Thr
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Gly Ala Leu Leu Gln Gln Gln Gln Ala Ser Gln Val Lys Gly Ala Leu
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Cys Phe Thr Gly Ile Ala Thr Leu Leu Arg Pro Gly Gln Gly Gln Gln
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Pro Gly Gln Gly Gln Gln Pro Gly Gln Gly Arg Pro Pro Met Pro Arg
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Gly Arg Pro Asn Ala Arg Pro Arg Pro Gly Pro Val Pro Gly Thr Pro
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Thr Val Ser Ser Pro Gly Ser Ser Pro Gly Ser Ser Pro Gly Ile Ser
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145 150 155 160
Phe Gly Ser Ser Leu Gly Ser Ser Ile Gly Ser Pro Pro Ala Thr Ser
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Gly Ser Tyr Gly Pro Leu Ser Met Gly Leu Gly Ala Pro Arg Arg Phe
50 55 60

Pro Gly Asp Leu Arg Leu Ile Ser Glu Pro Thr Ser Arg Leu Pro Val
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35 40 45
Gly Tyr Pro Gly Leu Thr Ala Ser Ile Gly Gly Glu Val Gly Ala Arg
50 55 60
Leu Gly Gly Arg Ala Gly Val Gly Val Ser Ser Tyr Gly Tyr Gly Tyr
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Pro Ser Trp Gly Tyr Pro Tyr Gly Gly Tyr Gly Gly Tyr Gly Gly Tyr
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Tyr Pro Gly Tyr Tyr Gly Tyr Tyr Tyr Pro Ser Gly Tyr Gly Gly Gly
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tggccctcgt ggatacccag ggcttaccgc atcgattgga ggcgaagtgg gtgcacgact 240

cggtggtcgt gccggtgtgg gagtgagcag ctacggctat ggttaccctt catggggcta 300

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Gly Tyr Pro Gly Leu Thr Ala Ser Ile Gly Gly Glu Val Gly Ala Arg
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Leu Gly Gly Arg Ala Gly Val Gly Val Ser Ser Tyr Gly Tyr Gly Tyr
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Pro Ser Trp Gly Tyr Pro Tyr Gly Gly Tyr Gly Gly Tyr Gly Gly Tyr
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Leu Gly Gly Arg Ala Gly
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<211> 64
<212> PRT
<213> Rhipicephalus appendiculatus

<220>
<221> 64trp4

<400> 20
Ala Gly Val Gly Val Ser Ser Tyr Gly Tyr Gly Tyr Pro Ser Trp Gly
1 5 10 15
Tyr Pro Tyr Gly Gly Tyr Gly Gly Tyr Gly Gly Tyr Gly Gly Tyr Gly
20 25 30
Gly Tyr Asp Gln Gly Phe Gly Ser Ala Tyr Gly Gly Tyr Pro Gly Tyr
35 40 45
Tyr Gly Tyr Tyr Tyr Pro Ser Gly Tyr Gly Gly Gly Tyr Gly Gly Ser
50 55 60

<210> 21
<211> 133
<212> PRT

<213> Rhipicephalus appendiculatus

<220>

<221> 64trp5

<400> 21

Met Lys Ala Phe Phe Val Leu Ser Leu Leu Ser Thr Ala Ala Leu Thr
1 5 10 15
Asn Ala Ala Arg Ala Gly Arg Leu Gly Ser Asp Leu Asp Thr Phe Gly
20 25 30
Arg Val His Gly Asn Leu Tyr Ala Gly Ile Glu Arg Ala Gly Pro Arg
35 40 45
Gly Tyr Pro Gly Leu Thr Ala Ser Ile Gly Gly Glu Val Gly Ala Arg
50 55 60
Leu Gly Gly Arg Ala Gly Val Gly Val Ser Ser Tyr Gly Tyr Gly Tyr
65 70 75 80
Pro Ser Trp Gly Tyr Pro Tyr Gly Gly Tyr Gly Gly Tyr Gly Gly Tyr
85 90 95
Gly Gly Tyr Gly Gly Tyr Asp Gln Gly Phe Gly Ser Ala Tyr Gly Gly
100 105 110
Tyr Pro Gly Tyr Tyr Gly Tyr Tyr Tyr Pro Ser Gly Tyr Gly Gly Gly
115 120 125
Tyr Gly Gly Ser Tyr
130

CLAIMS:

1. Use of an immunogenic 64p protein consisting of the amino acid sequence as set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, or 14, an immunogenic fragment thereof or an immunogenic homologue of said 64p protein or fragment having at least 50% identity thereto, or a plasmid DNA vector encoding said protein, fragment or homologue; for the preparation of a vaccine composition for the prevention of tick-borne encephalitis (TBE) or to reduce the transmission of the TBE virus to uninfected ticks of for both; wherein said vaccine composition is for administration to animals.

2. The use according to claim 1, wherein said fragment is as set forth in SEQ ID NO: 21 with or without a His Tag, or a homologue thereof having at least 50% identity to said fragment.

3. The use according to claim 2, wherein said fragment is SEQ ID NO: 21 or a homologue thereof having at least 50% identity to said fragment.

4. The use according to any one of claims 1 to 3, wherein said 64p protein, fragment thereof or homologue is expressed in recombinant form.

5. The use according to any one of claims 1 to 3, wherein said vaccine composition additionally comprises a second active agent, wherein said second active agent is an immunogenic protein, the 64p protein, fragments or homologues of the 64p protein, vaccines against infectious diseases or vaccines against TBE.

6. The use according to claim 5, wherein said second active agent is derived from a blood-feeding ectoparasite.

7. The use according to claim 6, wherein said second active agent is an immunogenic 64p protein, an immunogenic fragment thereof or an immunogenic homologue of said protein or fragment having at least 50% identity thereto.

8. The use according to claim 1, wherein said vaccine composition comprises the combination of SEQ ID NO: 21 and SEQ ID NO: 21 with a His tag.

9. The use according to any one of claims 1 to 3 or 6 to 8, wherein said 64p protein or fragment thereof or homologue is fused to another molecule.

10. The use according to claim 9, wherein said another molecule is a label, a toxin, a bioactive molecule or an immunogenic molecule.

11. The use according to claim 9, wherein said vaccine composition further comprises an adjuvant.

12. A vaccine composition comprising immunogenic 64p protein consisting of the amino acid sequence as set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, or 14, an immunogenic fragment thereof or an immunogenic homologue of said 64p protein or fragment having at least 50% identity thereto, or a plasmid DNA vector encoding said protein, fragment or homologue; for use in the prevention of tick-borne encephalitis (TBE) or reduction in transmission of the TBE virus to uninfected ticks of for both; wherein said vaccine composition is for administration to animals.

13. The vaccine composition according to claim 12, wherein said fragment is as set forth in SEQ ID NO: 21 with or without a His Tag, or, a homologue thereof having at least 50% identity to said fragment.

14. The vaccine composition according to claim 13, wherein said fragment is as set forth in SEQ ID NO: 21 or a homologue thereof having at least 50% identity to said fragment.

15. The vaccine composition according to any one of claims 12 to 14, wherein said 64p protein, fragment thereof or homologue is expressed in recombinant form.

16. The vaccine composition according to claim 15, wherein said vaccine composition additionally comprises a second active agent, wherein said second active agent is an immunogenic protein, the 64p protein, fragments or homologues of the 64p protein, vaccines against infectious diseases or vaccines against TBE.

17. The vaccine composition according to claim 16, wherein said second active agent is derived from a blood-feeding ectoparasite.

18. The vaccine composition according to claim 17, wherein said second active agent is an immunogenic 64p protein, an immunogenic fragment thereof or an immunogenic homologue of said protein or fragment having at least 50% identity thereto.

19. The vaccine composition according to claim 12, wherein said vaccine composition comprises the combination of SEQ ID NO: 21 and SEQ ID NO: 21 with a His tag.

20. The vaccine composition according to claim 19, wherein said 64p protein or fragment thereof or homologue is fused to another molecule.

21. The vaccine composition according to claim 20, wherein said another molecule is a label, a toxin, a bioactive molecule or an immunogenic molecule.

22. The vaccine composition according to any one of claims 16 to 21, wherein said vaccine composition further comprises an adjuvant.

FIG. 1

```
Clone21 1   AAACCAAGGCAGGACACAGCAGCCATGAAGGCCTTCGTTGCAGTCGCCCTTTTGTCTGCA   60
                                      M  K  A  F  V  A  V  A  L  L  S  A
Clone33 1   CGGACGCACACTCCTGCAGGAAGGTCATCTAGTTCCGCCAACATGAAGCTGCTCTGTGCA   60
                                                  M  K  L  L  C  A
CemA    1   GATCGGCACGAGGTCAAGGGAGCCCTCCTTCAGCAACAACAAGCATCGCAGGTTAAGGGA   60
             D  R  H  E  V  K  G  A  L  L  Q  Q  Q  Q  A  S  Q  V  K  G
Clone24 1   GGAAGTAGCGAGCATCCGCACTGGGGTCTTTTTGGCTGCATTTGCTTTTCTTCTTTCAGC   60
            E  V  A  S  I  R  T  G  V  F  L  A  A  F  A  F  L  L  S  A
Clone68 1   GGCTTCGGCAGCCCACTCAGCGGTTTCGGCAGCCCACTCAGCGGTTTCGGCAGCCCACTC   60
            G  F  G  S  P  L  S  G  F  G  S  P  L  S  G  F  G  S  P  L
Clone64 1   GGAGATCACCTGCTTGCAAAGGACAACGTCCTAACACAGCCGCAAAATGAAAGCTTTCTT   60
                                                  M  K  A  F  F
CloneI  1   ACGGACTAGGTTTCGCTGGCGTCCCTCTTATTGGCGGATACGGCTACGGTCCTTTCGTAG   60
            G  L  G  F  A  G  V  P  L  I  G  G  Y  G  Y  G  P  F  V  G
```

FIG. 1 (CONTD.)

```
Clone21 61   GTTTCCGTGGCACATGCTGCCCTCAAGACTGACGTAGCCAGTGGACCTGCCGGTTCTGGT   120
             V  S  V  A  H  A  A  L  K  T  D  V  A  S  G  P  A  G  S  G
Clone33 61   CTAGCCCTCGTTGCCCTTGGACTTCCATTCGGCAGCGCTTACCTTGGTGGCTTCGGCGGC   120
             L  A  L  V  A  L  G  L  P  F  G  S  A  Y  L  G  G  F  G  G
CemA    61   GCCCTCAAGGGAGCAATCAAGGGTGGTCTTCTTCAGCAACAAGCCCAATCCCAAGTCCAA   120
             A  L  K  G  A  I  K  G  G  L  L  Q  Q  Q  A  Q  S  Q  V  Q
Clone24 61   GATCCATAACAATGGCCAGTCATGTGTAGATGCAGCCCCCACTCGACGTCCTATGCCATC   120
             I  H  N  N  G  Q  S  C  V  D  A  A  P  T  R  R  P  M  P  S
Clone68 61   AGCGGCTTCGGCAGCCCACTCAGCGGATTCGGTAGCCCACTCAGCGGATTCGGTAGCCCA   120
             S  G  F  G  S  P  L  S  G  F  G  S  P  L  S  G  F  G  S  P
Clone64 61   CGTTCTTTCCCTTCTTTCAACCGCCGCACTGACGAATGCAGCAAGGGCTGGTCGTCTTGG   120
             V  L  S  L  L  S  T  A  A  L  T  N  A  A  R  A  G  R  L  G
CloneI  61   GAGCCTTCGCGTACGGCTTGTGGGGTGGCCTCGGTGGCTATGGCTACCCTGCCTTCGGAC   120
               A  F  A  Y  G  L  W  G  G  L  G  G  Y  G  Y  P  A  F  G  L
```


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FIG. 1(CONTD.)

```
Clone21  121  GCACTAAGTCTAGGAGTTGGAGGCTTCCCGTCCGGTGCTTCGCTTGGCAGCCTTAGTGGC   180
              A  L  S  L  G  V  G  G  F  P  S  G  A  S  L  G  S  L  S  G
Clone33  121  CTCGGTGGTTGGGGAGGCGGTCTCGGAGCCATCTTTGGCCCAGGAGCTTATCCCGGTTTC   180
              L  G  G  W  G  G  G  L  G  A  I  F  G  P  G  A  Y  P  G  F
CemA     121  GGAGCTCTTAAGGGAGCCGTCAAGGGAGCCCTCCTTCAGCAACAACAGGCATCACAGGTC   180
              G  A  L  K  G  A  V  K  G  A  L  L  Q  Q  Q  Q  A  S  Q  V
Clone24  121  TCCTCCTGGATGTGCTGGTCCTGGCTGTTTTACTGGTATTGCTACTCTTCTAAGACCTGG   180
              P  P  G  C  A  G  P  G  C  F  T  G  I  A  T  L  L  R  P  G
Clone68  121  CTCAGCGGATTCGGTAGCCCATTCGGCAGCTACGGTCCCCTGTCCATGGGTCTCGGAGCC   180
              L  S  G  F  G  S  P  F  G  S  Y  G  P  L  S  M  G  L  G  A
Clone64  121  AAGCGACCTGGATACATTTGGAAGGGTACACGGTAACCTATATGCCGGCATCGAAAGAGC   180
              S  D  L  D  T  F  G  R  V  H  G  N  L  Y  A  G  I  E  R  A
CloneI   121  TCTCCTGGGTTCCACATGGTTTTGGAGGCTTTGGAGCTTCTCCGTCTGCTGCTGGTTTCC   180
              S  W  V  P  H  G  F  G  G  F  G  A  S  P  S  A  A  G  F  R
```

FIG. 1(CONTD.)

```
Clone21 181 GTAACCCTCTCTGGTGCTGGCTCTTCCGTGTCTGGCCGCCCTGGATCCCCTGGATCGGCT   240
            V  T  L  S  G  A  G  S  S  V  S  G  R  P  G  S  P  G  S  A
Clone33 181 TATGGCCTTAACAGCGTGCACCTGTTGGGCGGCAGGTTCCACCATCTCTTCGGGCGATTC   240
            Y  G  L  N  S  V  H  L  L  G  G  R  F  H  H  L  F  G  R  F
CemA    181 AAGGGAGCCCTCAAGGGAGCCATCAAGGTCTGTCTCCTTCATCAGCAAGCCCAATCCCAA   240
            K  G  A  L  K  G  A  I  K  V  C  L  L  H  Q  Q  A  Q  S  Q
Clone24 181 TCAAGGACAGCAACCTGGTCAAGGACAGCAACCTGGTCAAGGGCGTCCTCCAATGCCACG   240
            Q  G  Q  Q  P  G  Q  G  Q  Q  P  G  Q  G  R  P  P  M  P  R
Clone68 181 CCCAGGAGATTCCCCGGCGACCTCCGCCTCATCTCTGAGCCCACCTCCCGCCTTCCCGTT   240
            P  R  R  F  P  G  D  L  R  L  I  S  E  P  T  S  R  L  P  V
Clone64 181 TGGCCCTCGTGGATACCCAGGGCTTACCGCATCGATTGGAGGCGAAGTGGGTGCACGACT   240
            G  P  R  G  Y  P  G  L  T  A  S  I  G  G  E  V  G  A  R  L

CloneI  181 GCTCGCTTTGGAGCCTCTT                                          199
            S  L  W  S  L

Clone21 241 GGTCCTAGCTCTGGACCCGCAGTGTCG                                  267
            G  P  S  S  G  P  A  V  S
Clone33 241 CCGCCACCACCCGGTATTGGAGCTGCTGAGCGCAGGGGAACCCTAAGCCCATACCCTCTT   300
            P  P  P  P  G  I  G  A  A  E  R  R  G  T  L  S  P  Y  P  L
CemA    241 TCCCAAGTTCAGGGAGCTCTTAAGGGAGCTG                              271
            S  Q  V  Q  G  A  L  K  G  A
Clone24 241 TCCAGGACCTGTTCCAGGAACATCTGGATCACCTCAAGGAAGACCCAATGGAGCACCTCG   300
            P  G  P  V  P  G  T  S  G  S  P  Q  G  R  P  N  G  A  P  R
Clone68 241 AGCGATGCCGTCTACACCGCTGTCGTCCAGCCCGTCACAAGCGCAGTGGTCCACACCGAG   300
```

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FIG. 1(CONTD.)

```
Clone33 301 GACATCAACACCGTCCAAGACCCCGTAACTGGCCACCCCATGGTACGCGTTGTCTACGGC  360
            D  I  N  T  V  Q  D  P  V  T  G  H  P  M  V  R  V  V  Y  G
Clone24 301 TCCAGGACCTGTTCCTGGAACATCTGGATCACCTCAAGGAAGACCTAACGCAAGACCTCG  360
            P  G  P  V  P  G  T  S  G  S  P  Q  G  R  P  N  A  R  P  R
Clone68 301 GGTCCCCATGTCACCGGCCAAGTACAGGAACACGTTGCAATCTAAGCTTTTCTAACCGCA  360
            G  P  H  V  T  G  Q  V  Q  E  H  V  A  I
Clone64 301 TCCGTATGGTGGATACGGTGGATACGGTGGATACGGTGGATACGGTGGATATGATCAGGG  360
            P  Y  G  G  Y  G  G  Y  G  G  Y  G  G  Y  G  G  Y  D  Q  G

Clone33 361 GGCGTCTTGCGGGAGCGCCTCGTACCCGTACCAGTCCCAATTCCACAGGATGTGCCTGTC  420
            G  V  L  R  E  R  L  V  P  V  P  V  P  I  P  Q  D  V  P  V
Clone24 361 TCCAGGACCTGTTCCTGGAACACCAACTGTATCCTCTCCCGGATCATCTCCTGGGTCATC  420
            P  G  P  V  P  G  T  P  T  V  S  S  P  G  S  S  P  G  S  S
Clone68 361 AGCTATATTACGACGGATTAGTCAACACAGTCATCTTAAGCAAATGTATCTAAAATAAAA  420

Clone64 361 TTTTGGCTCTGCATACGGCGGCTACCCCGGCTACTATGGCTACTACTATCCCAGTGGCTA  420
            F  G  S  A  Y  G  G  Y  P  G  Y  Y  G  Y  Y  Y  P  S  G  Y
```

FIG. 1 (CONTD.)

```
Clone33 421 CCAGTCCCTATTCCAGTGCCCCAGCCATACCCAGTCCCACACCCACGACAAGTTCCATAC   480
            P  V  P  I  P  V  P  Q  P  Y  P  V  P  H  P  R  Q  V  P  Y
Clone24 421 TCCAGGAATATCTCTAGGAACGCCTCTAGGAACACCTCTAGGAACACCTCAAGGATCACC   480
            P  G  I  S  L  G  T  P  L  G  T  P  L  G  T  P  Q  G  S  P
Clone68 421 TTTATCTGCCT  431

Clone64 421 CGGTGGGGGCTACGGTGGTAGCTACGGTGGCAGCTACGGTGGTAGCTACACCTATCCCAA   480
            G  G  G  Y  G  G  S  Y  G  G  S  Y  G  G  S  Y  T  Y  P  N
```

```
Clone33 481 CCAGTGCCTAGTCCCTACCCCGTCCCAATCCACAGTAACACCGAAGTTCACAAGACCGAC   540
            P  V  P  S  P  Y  P  V  P  I  H  S  N  T  E  V  H  K  T  D
Clone24 481 TTTTGGATCATCTCTTGGATCATCGATAGGATCACCTCCTGCAACATCTCCTGGATCATC   540
            F  G  S  S  L  G  S  S  I  G  S  P  P  A  T  S  P  G  S  S
Clone64 481 CGTTCGGGCTTCAGCTGGTGCCGCAGCTTGAGCTTCTCCTTCAGCGTCACAGTAAGAAAT   540
            V  R  A  S  A  G  A  A  A  *
```

```
Clone33 541 GTCGTCGCCGCTACTCCAGGAGGACCAGTCCTGCTCGAGTCCGGTGTCACCGGCGTCAGG   600
            V  V  A  A  T  P  G  G  P  V  L  L  E  S  G  V  T  G  V  R
Clone24 541 TTCTCCGTCACCTCCTGGATCAGCGAATGTGAACCTGCTGGGTCCTCGACCAATTCGCGG   600
            S  P  S  P  P  G  S  A  N  V  N  L  L  G  P  R  P  I  R  G
Clone64 541 CATGGAGCACCCGATCGAGAAATACAGAGGTTCTCAAAAGCGTACGGGATGCCAACCAGC   600
```

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FIG. 1(CONTD.)

```
Clone33 601 CCAGGCGAACCAAGGGTCGTGGCCTAAGCTTGATCCAATAGAAAGTCATAACAATTTAGT   660
            P  G  E  P  R  V  V  A  *
Clone24 601 TCCTGGAAGGCATTGACGGGACCAGTTCTGCTGTGTATTCCTCCGTGCACAATGAGGGAA   660
            P  G  R  H
Clone64 601 AAGAAATTGCGCCGCAAAATGTTGAGAACAAATACAAGTTTTCTGTAAAAAAAAAA      656

Clone24 661 GGCATTGATGGGACCAGTTCTGCTGTGTATTTCTCCGTGCACAGTGAGGGAATCTATCAA   720

Clone24 721 TAGTGCAATAA                                                  731
```

FIG. 2

```
1   GGAGATCACCTGCTTGCAAAGGACAACGTCCTAACACAGCCGCAAAATGAAAGCTTTCTT   60
1                                                   M  K  A  F  F    5

                                                    P1/P7 ---------->

61  CGTTCTTTCCCTTCTTTCAACCGCCGCACTGACGAATGCAGCAAGGGCTGGTCGTCTTGG  120
6   V  L  S  L  L  L  S  T  A  A  L  T  N  A  A  R  A  G  R  L  G   25

121 AAGCGACCTGGATACATTTGGAAGGGTACACGGTAACCTATATGCCGGCATCGAAAGAGC  180
26  S  D  L  D  T  F  G  R  V  H  G  N  L  Y  A  G  I  E  R  A   45

181 TGGCCCTCGTGGATACCCAGGGCTTACCGCATCGATTGGAGGCGAAGTGGGTGCACGACT  240
46  G  P  R  G  Y  P  G  L  T  A  S  I  G  G  E  V  G  A  R  L   65
          <---------- P2

241 CGGTGGTCGTGCCGGTGTGGGAGTGAGCAGCTACGGCTATGGTTACCCTTCATGGGGCTA  300
66  G  G  R  A  G  V  G  V  S  S  Y  G  Y  G  Y  P  S  W  G  Y   85
       <----------P3----------P6---------->

301 TCCGTATGGTGGATACGGTGGATACGGTGGATACGGTGGATACGGTGGATATGATCAGGG  360
86  P  Y  G  G  Y  G  G  Y  G  G  Y  G  G  Y  G  G  Y  D  Q  G   105
                                                    P4 ---------->

361 TTTTGGCTCTGCATACGGCGGCTACCCCGGCTACTATGGCTACTACTATCCCAGTGGCTA  420
106  F  G  S  A  Y  G  G  Y  P  G  Y  Y  G  Y  Y  Y  P  S  G  Y   125

421 CGGTGGGGGCTACGGTGGTAGCTACGGTGGCAGCTACGGTGGTAGCTACACCTATCCCAA  480
126  G  G  G  Y  G  G  S  Y  G  G  S  Y  G  G  S  Y  T  Y  P  N   145
          <---------- P5

481 CGTTCGGGCTTCAGCTGGTGCCGCAGCTTGAGCTTCTCCTTCAGCGTCACAGTAAGAAAT  540
146  V  R  A  S  A  G  A  A  A  *                                155

541 CATGGAGCACCCGATCGAGAAATACAGAGGTTCTCAAAAGCGTACGGGATGCCAACCAGC  600

601 AAGAAATTGCGCCGCAAAATGTTGAGAACAAATACAAGTTTTCTGTAAAAAAAAAA  656
```

FIG. 3A

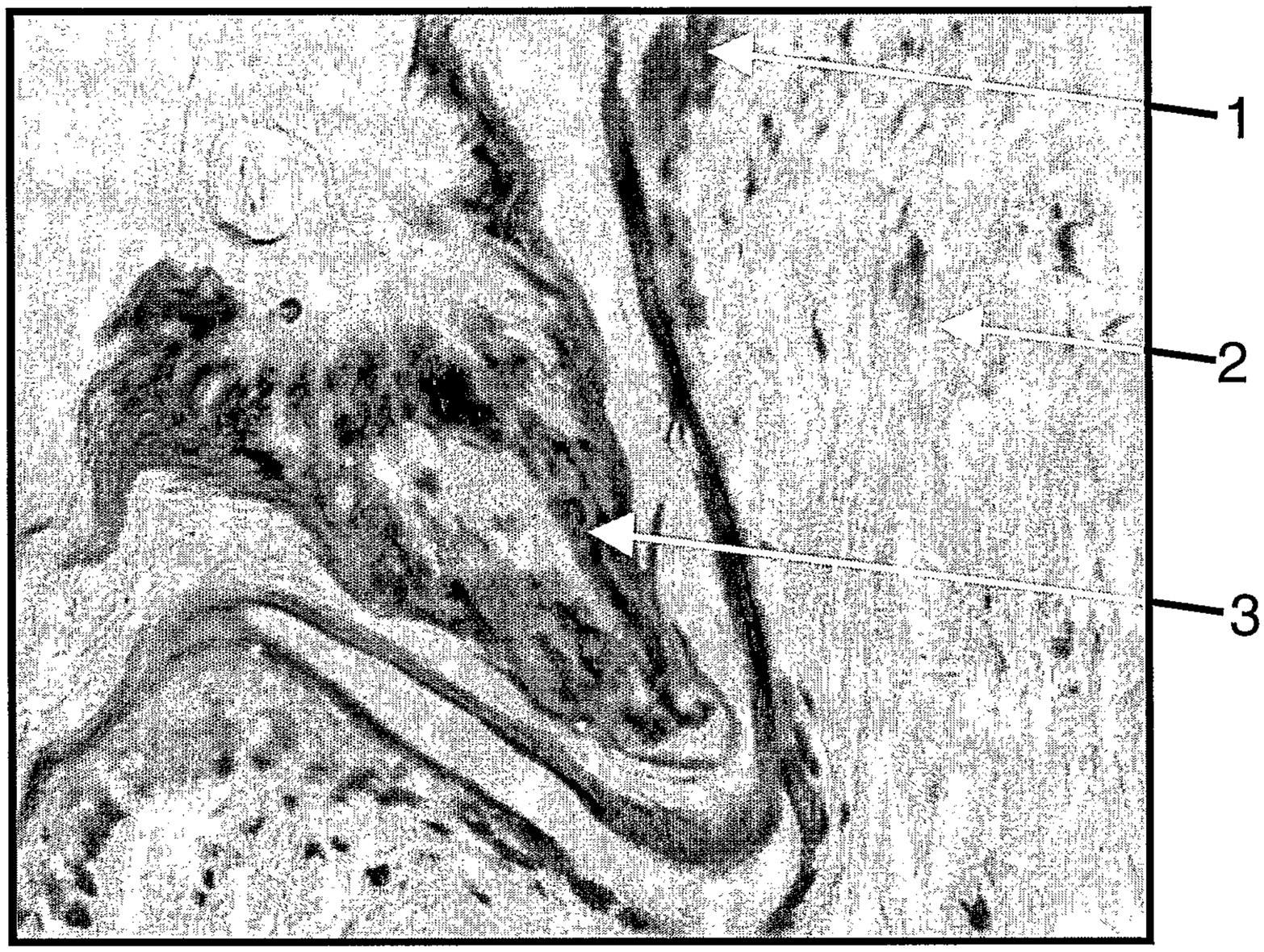


FIG. 3B

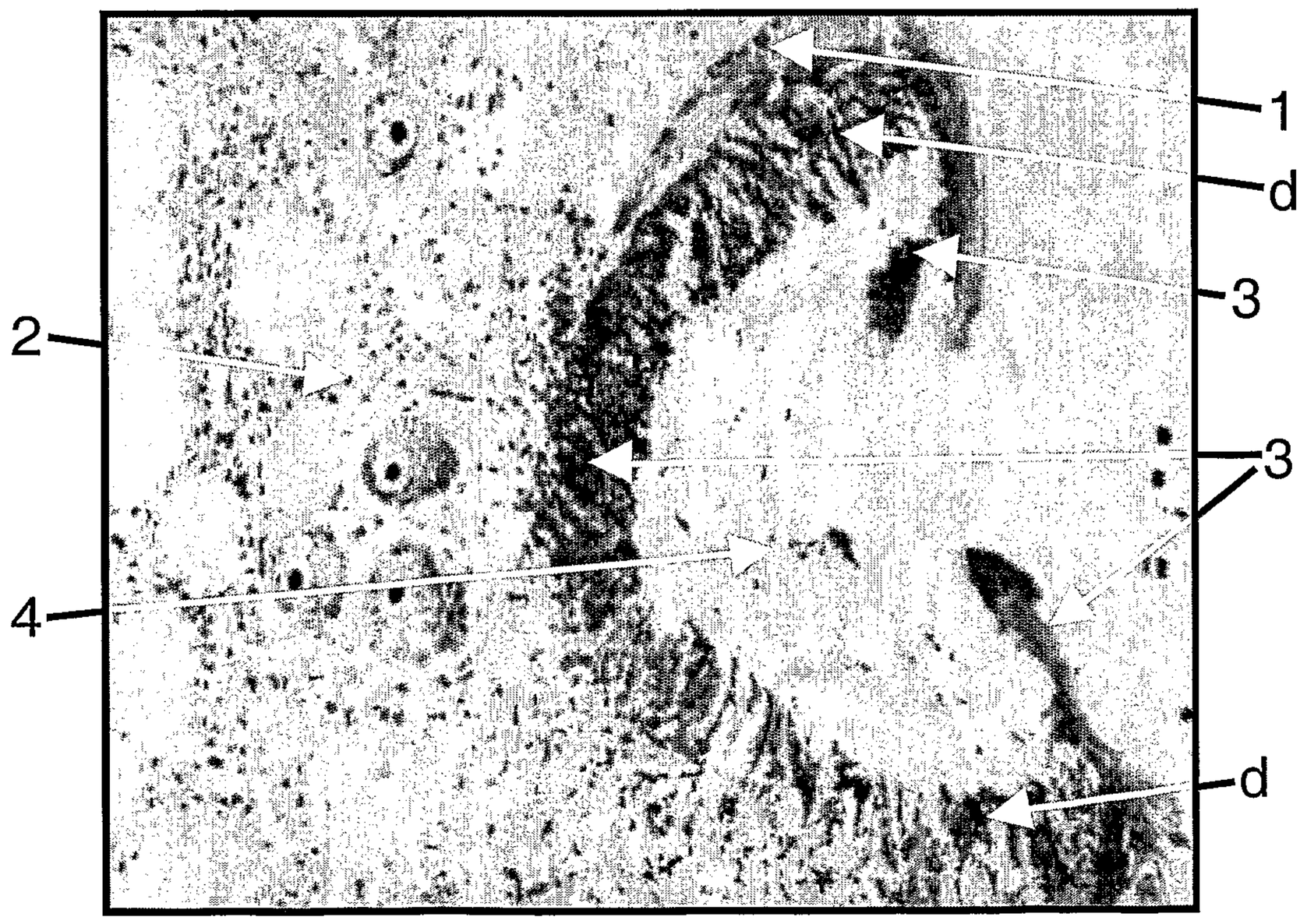


FIG. 3C

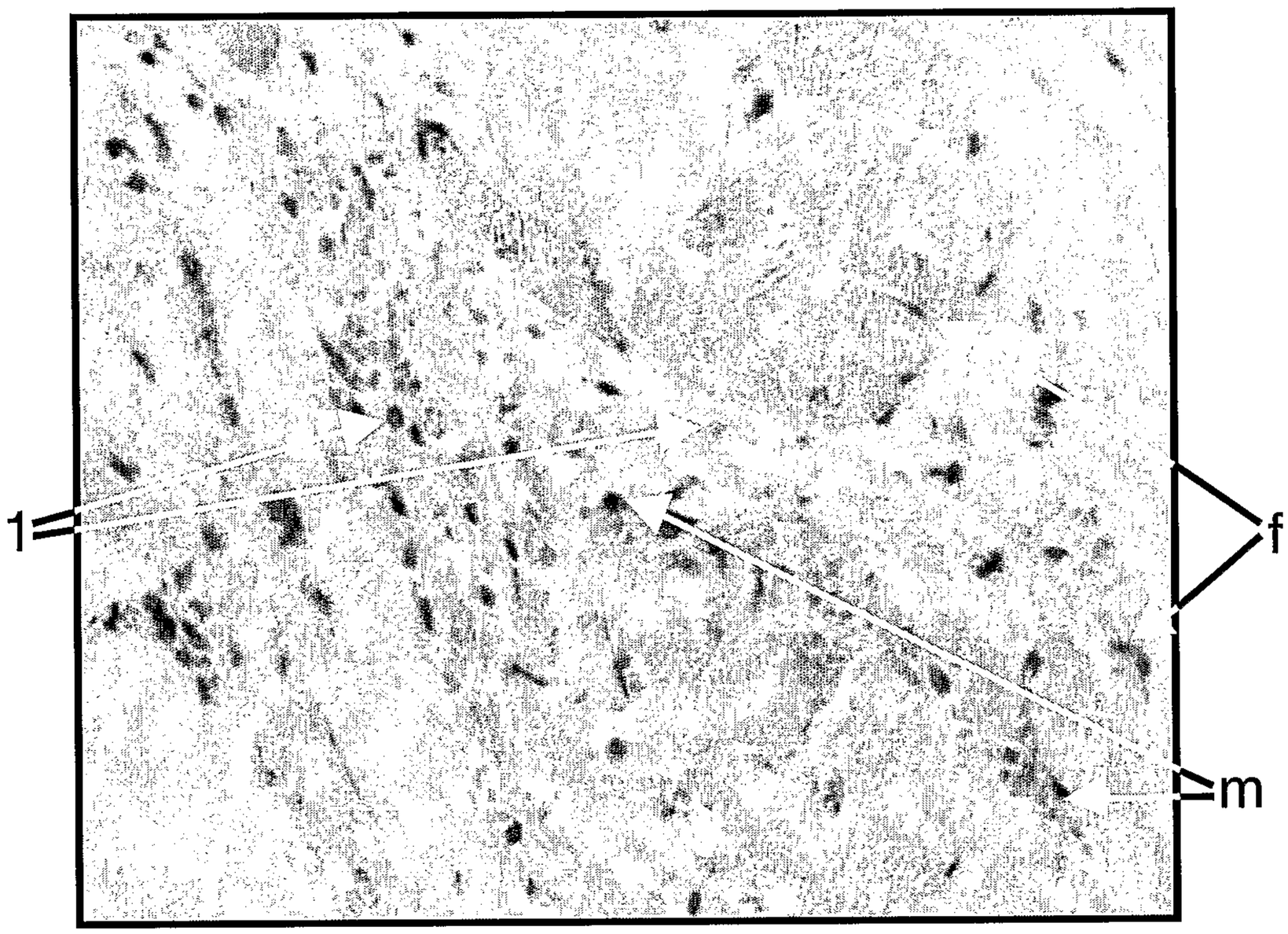


FIG. 3D

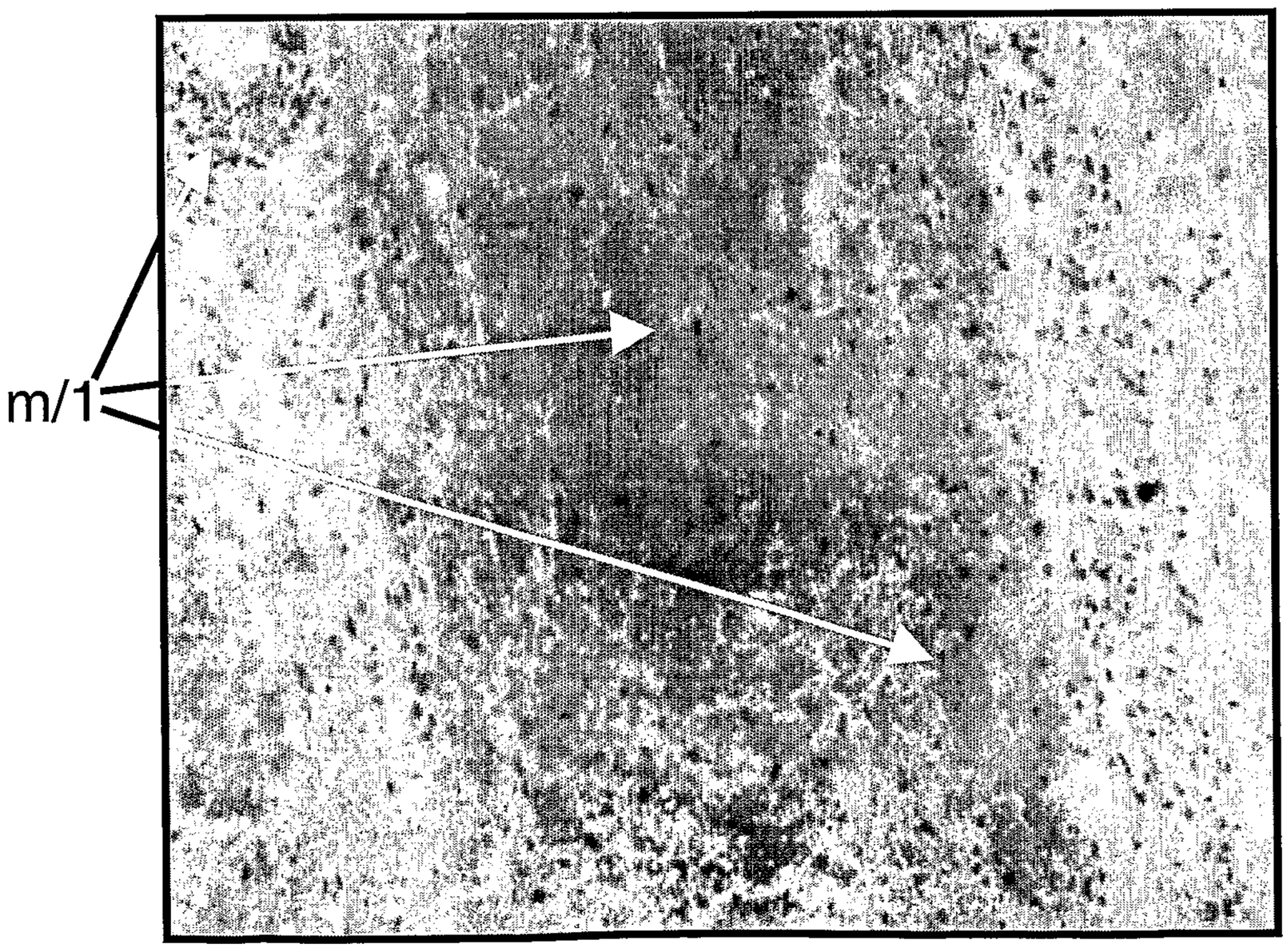


FIG. 4A

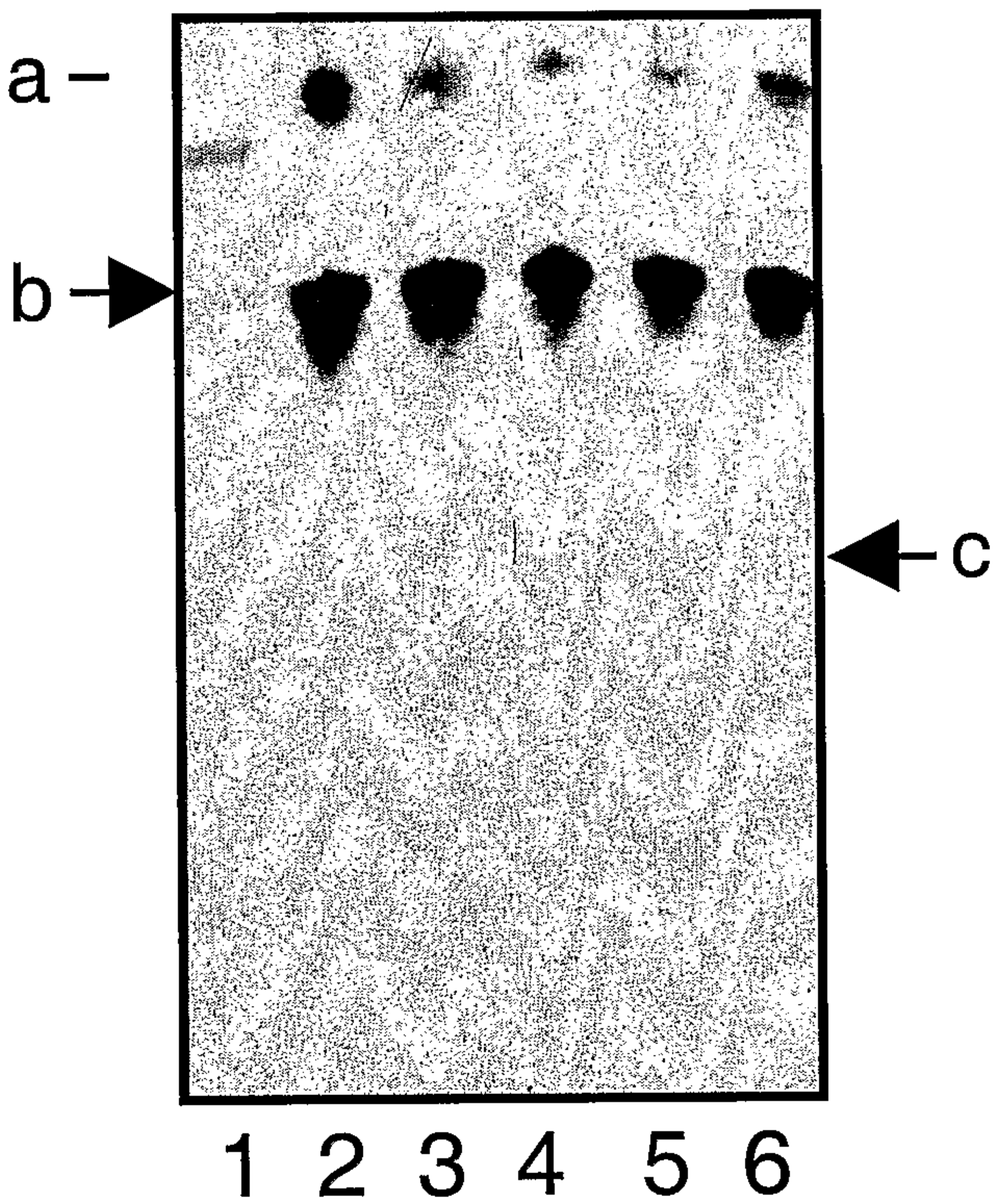


FIG. 4B

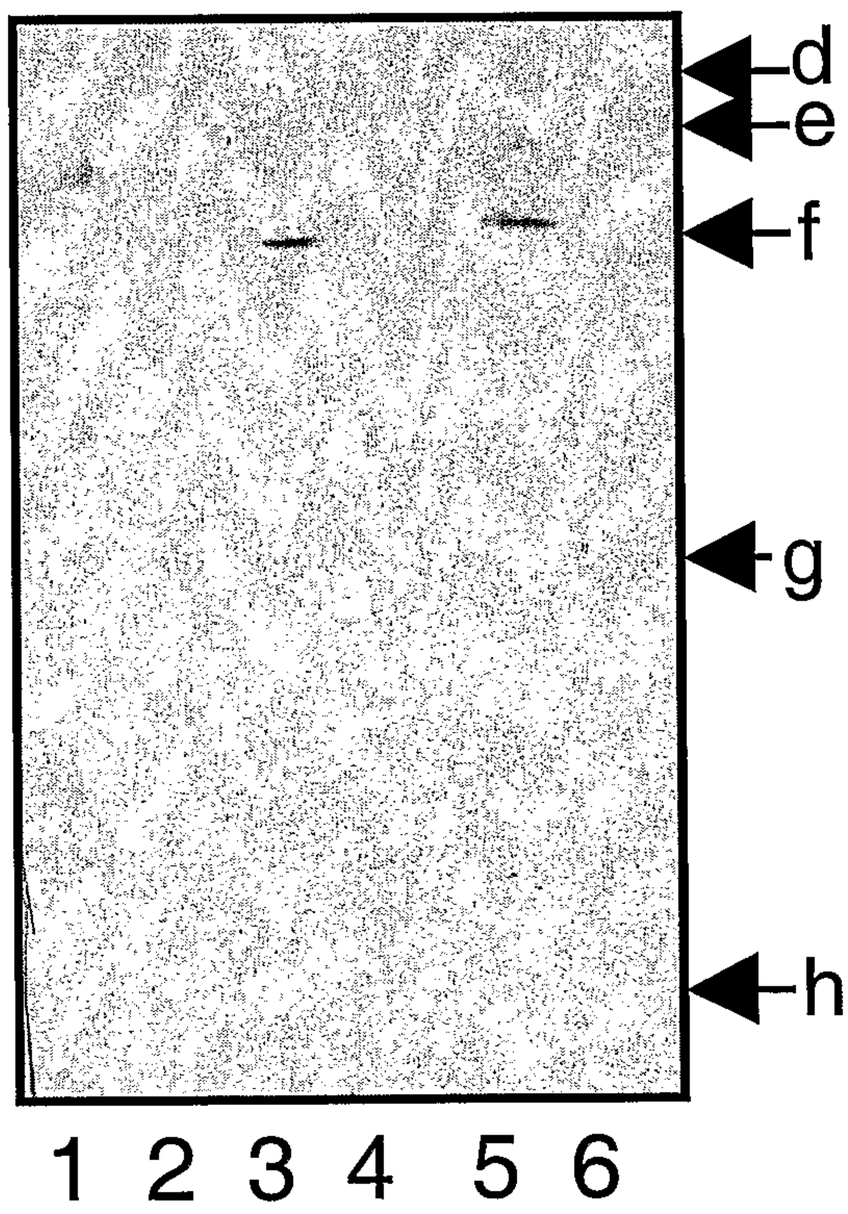


FIG. 4C

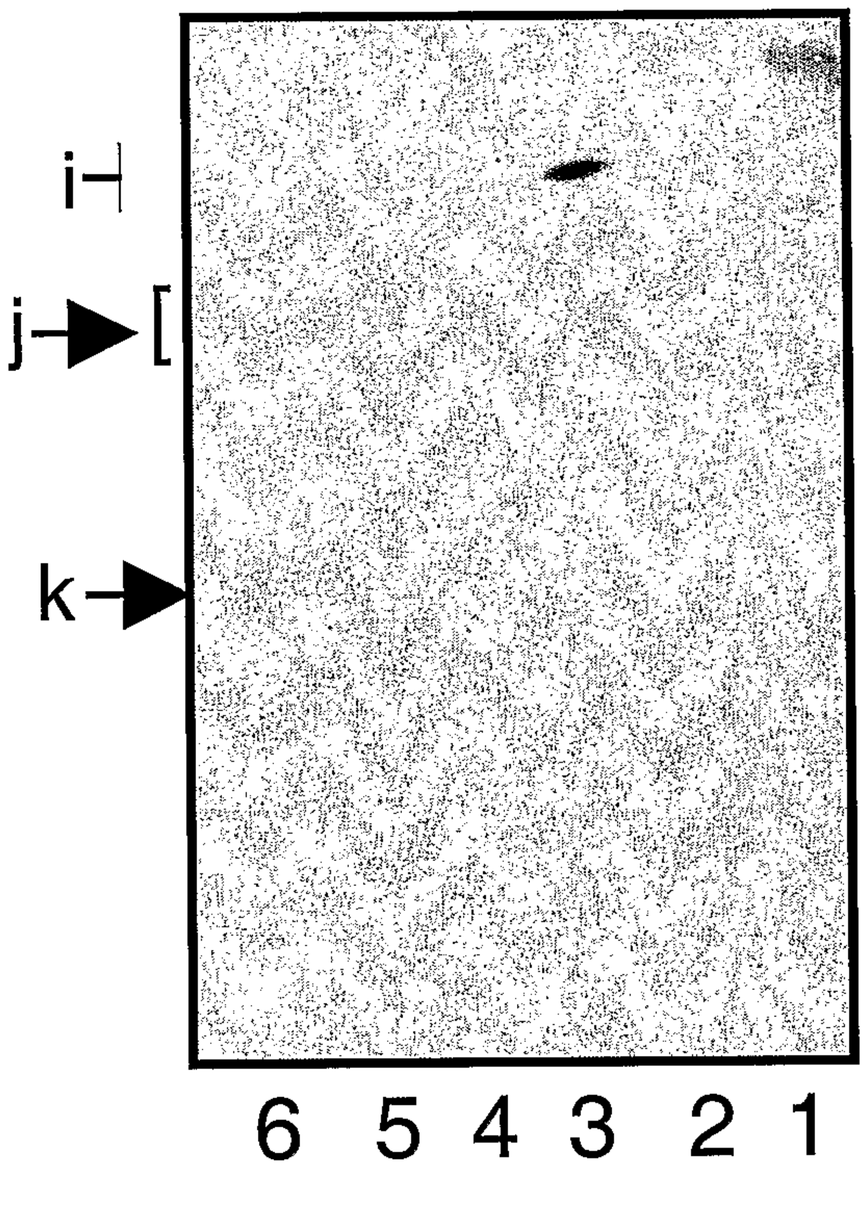


FIG. 4D

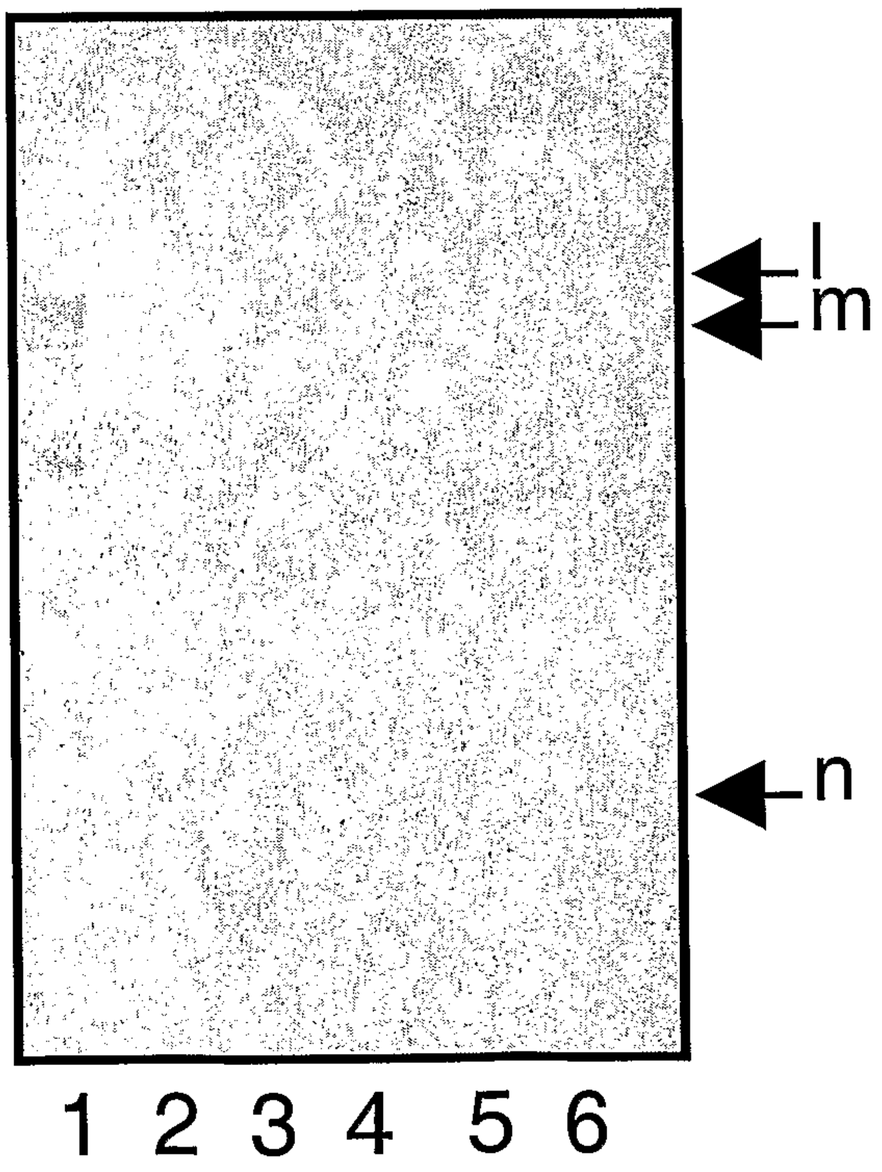


FIG. 4E

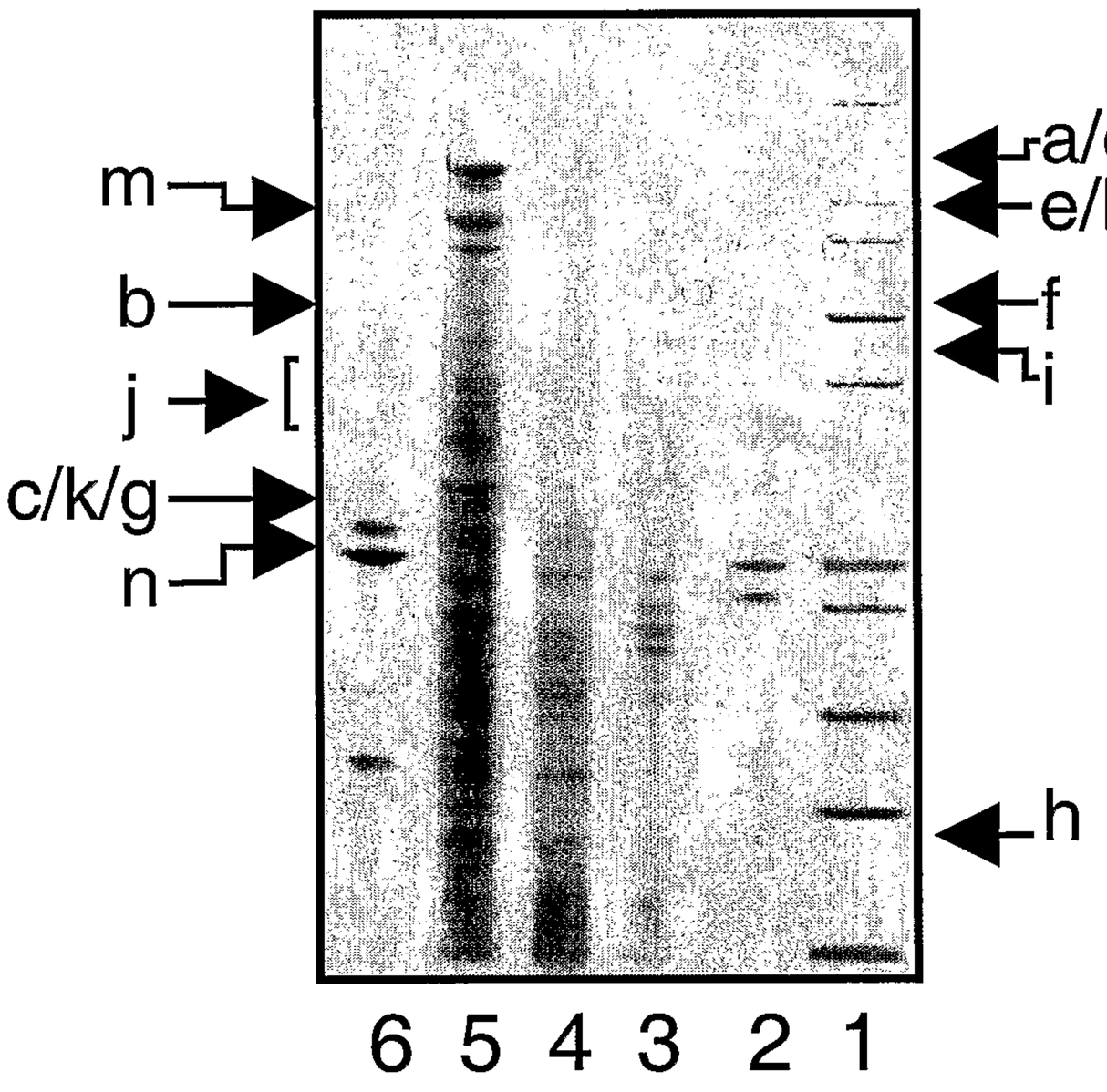


FIG. 5A

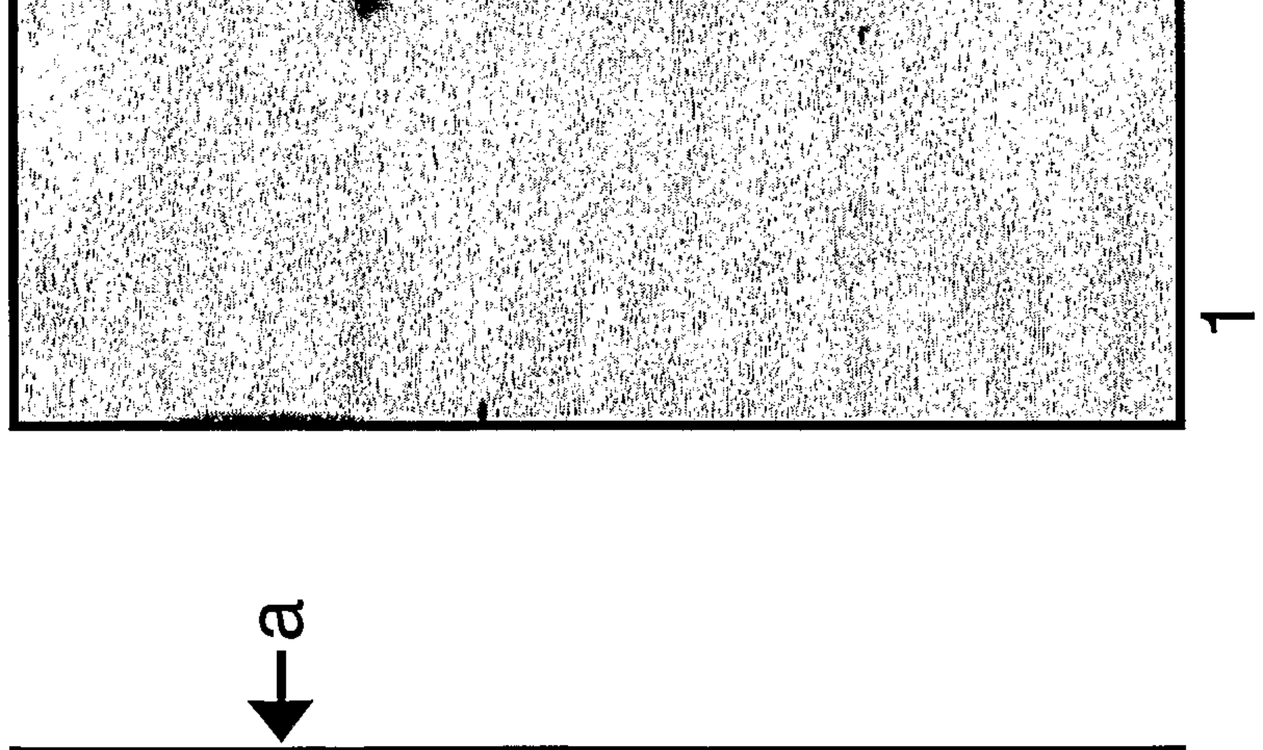


FIG. 5B

FIG. 5C

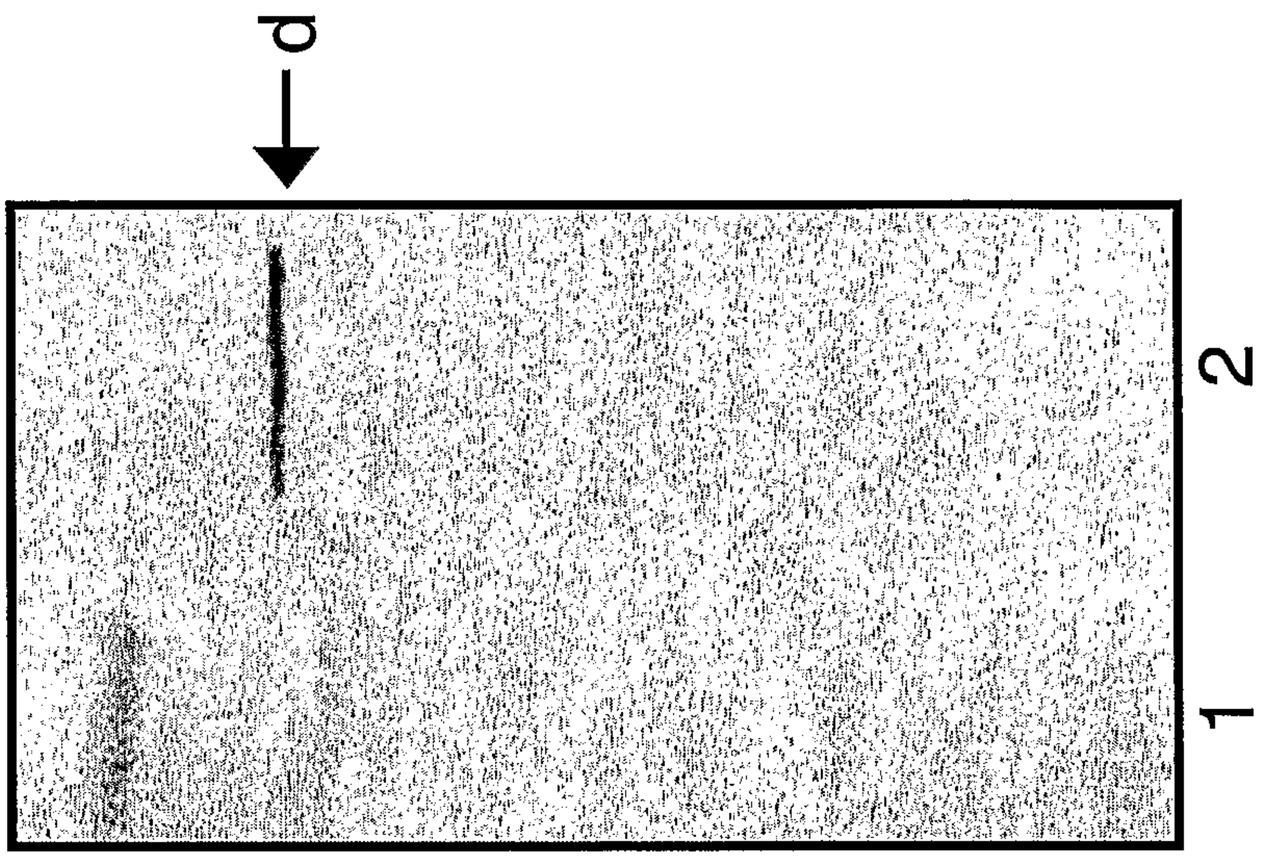


FIG. 5F

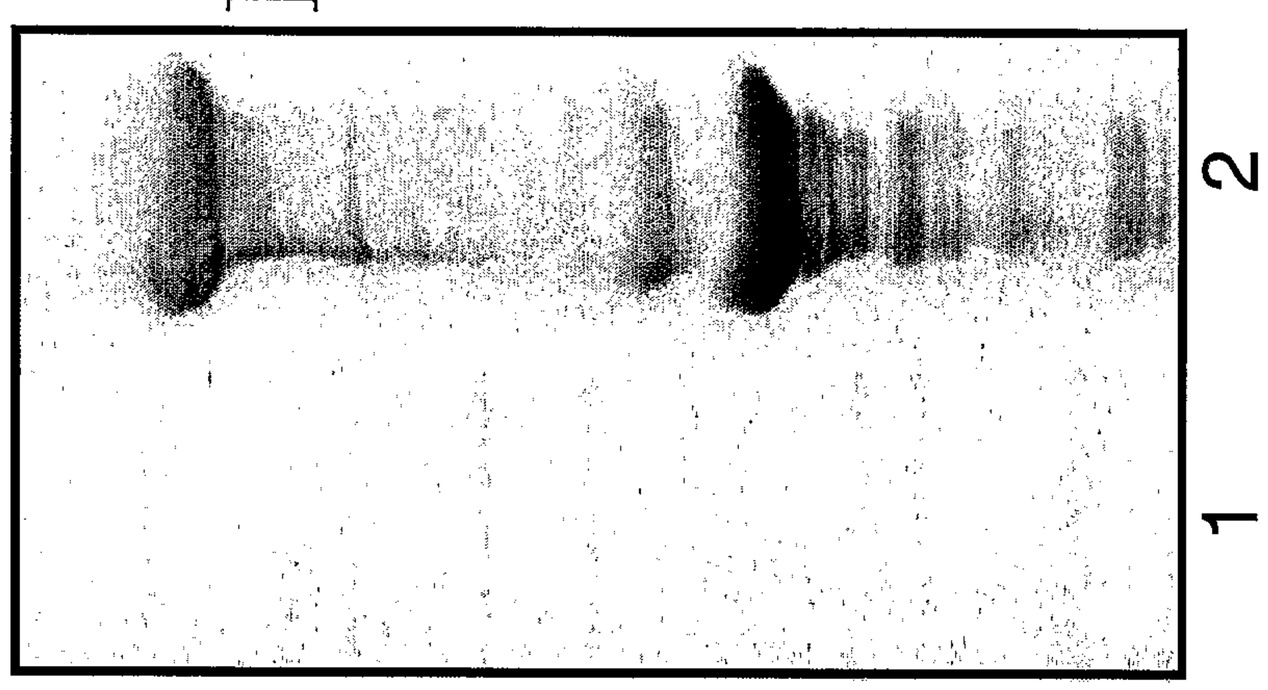


FIG. 5E

FIG. 5D

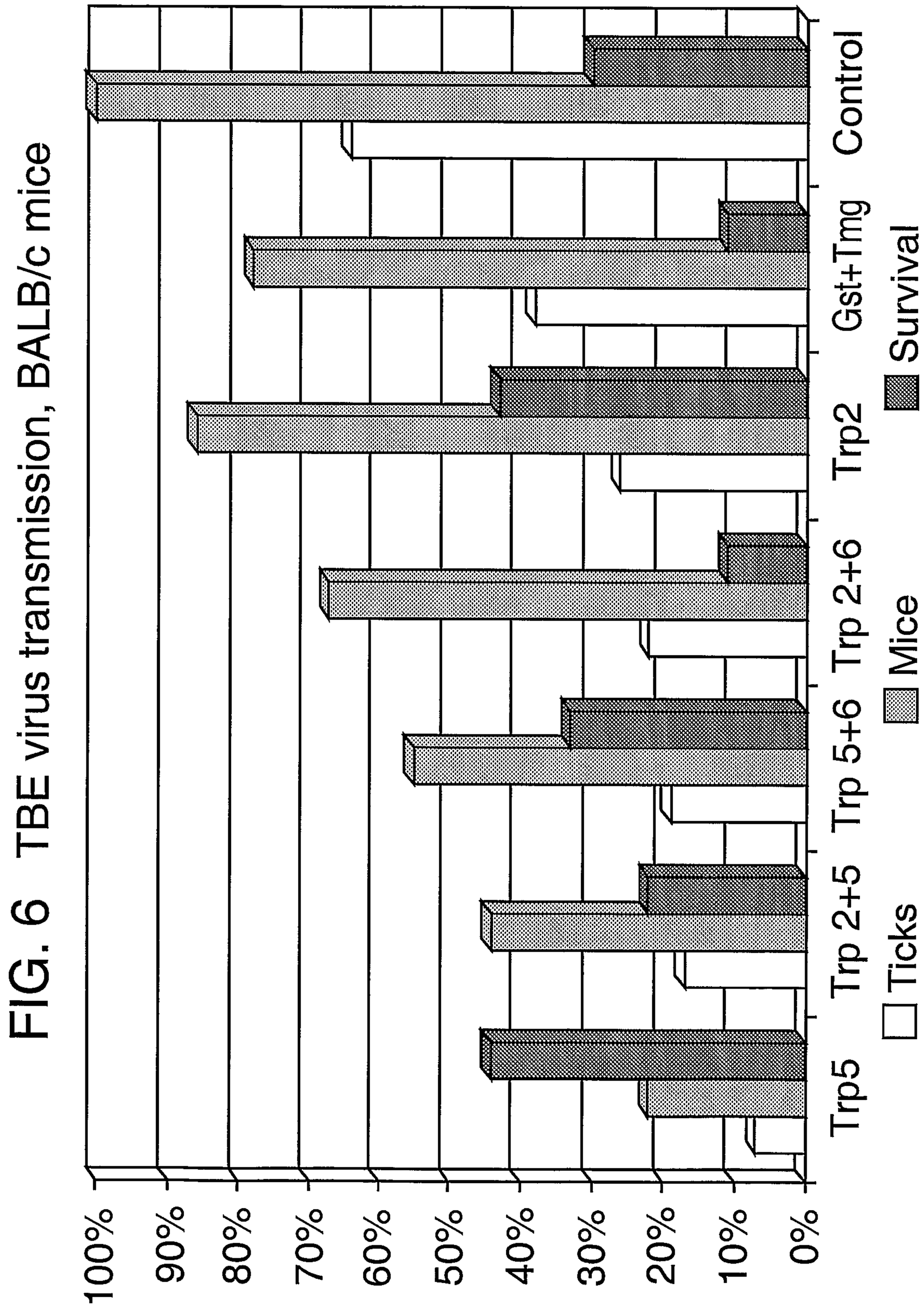


FIG. 6 TBE virus transmission, BALB/c mice
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Trp5
Trp 2+5
Trp 5+6
Trp 2+6
Trp2
Gst+Tmg
Control
Ticks
Mice
Survival

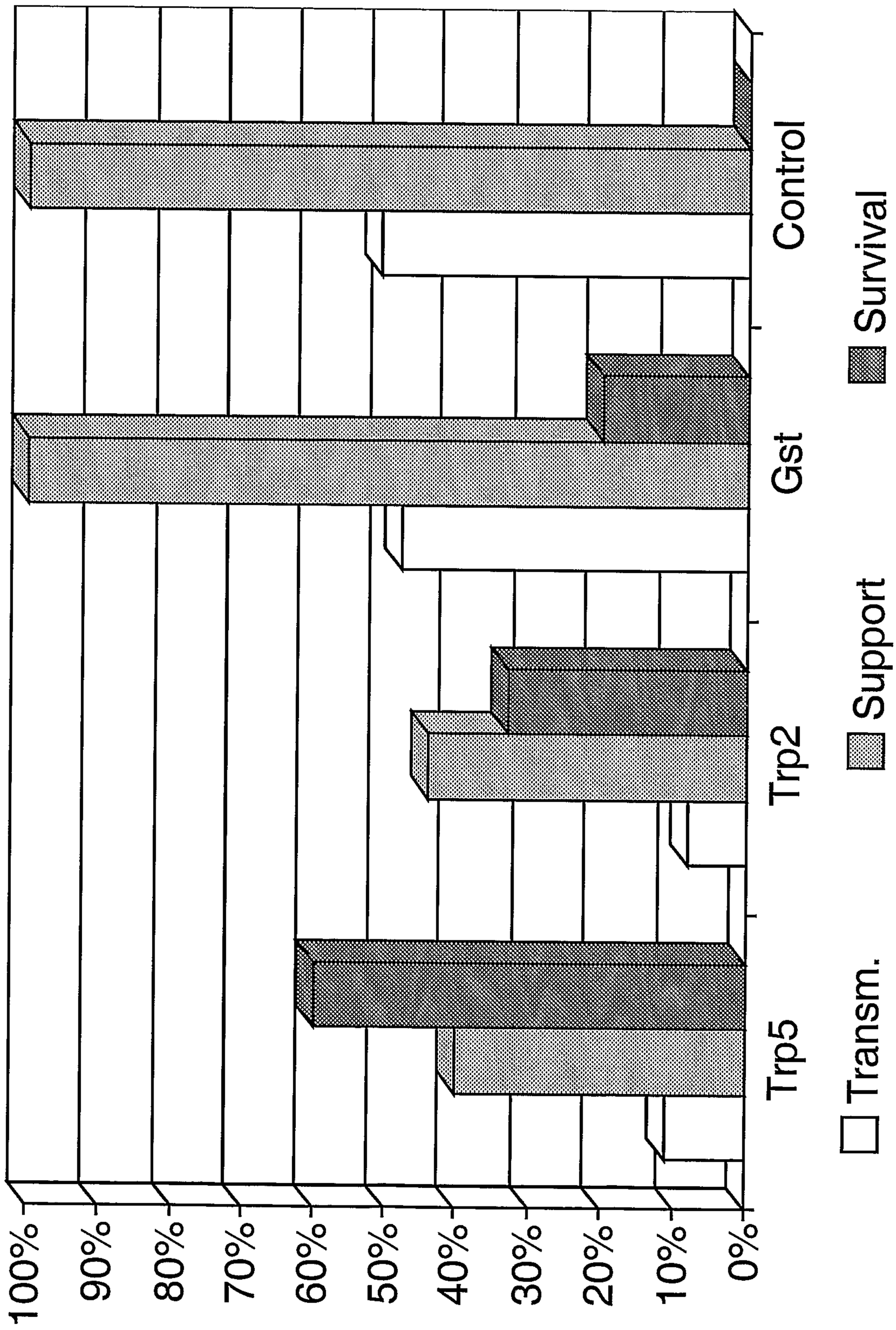


FIG. 7 TBE virus transmission, trial II

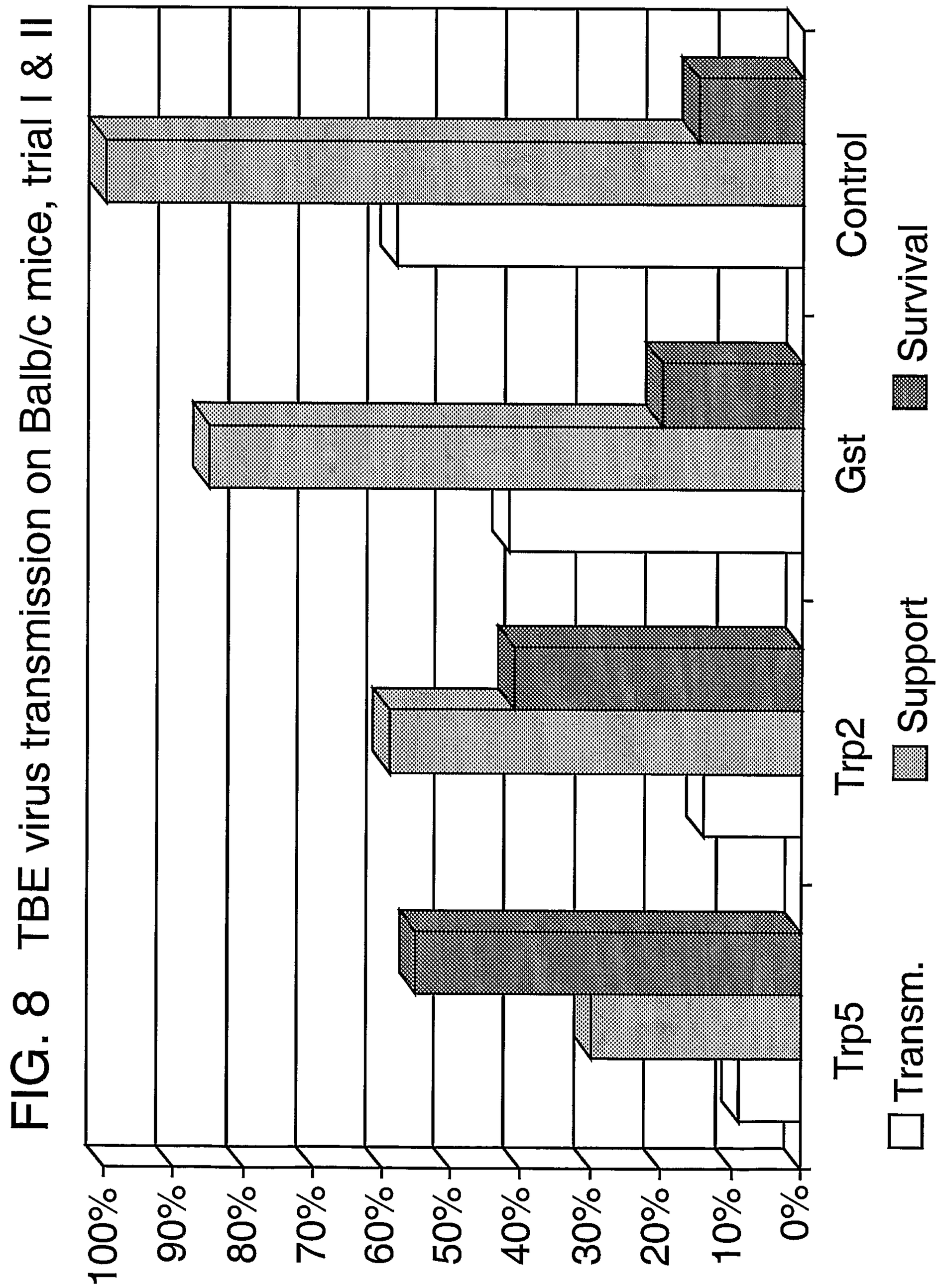
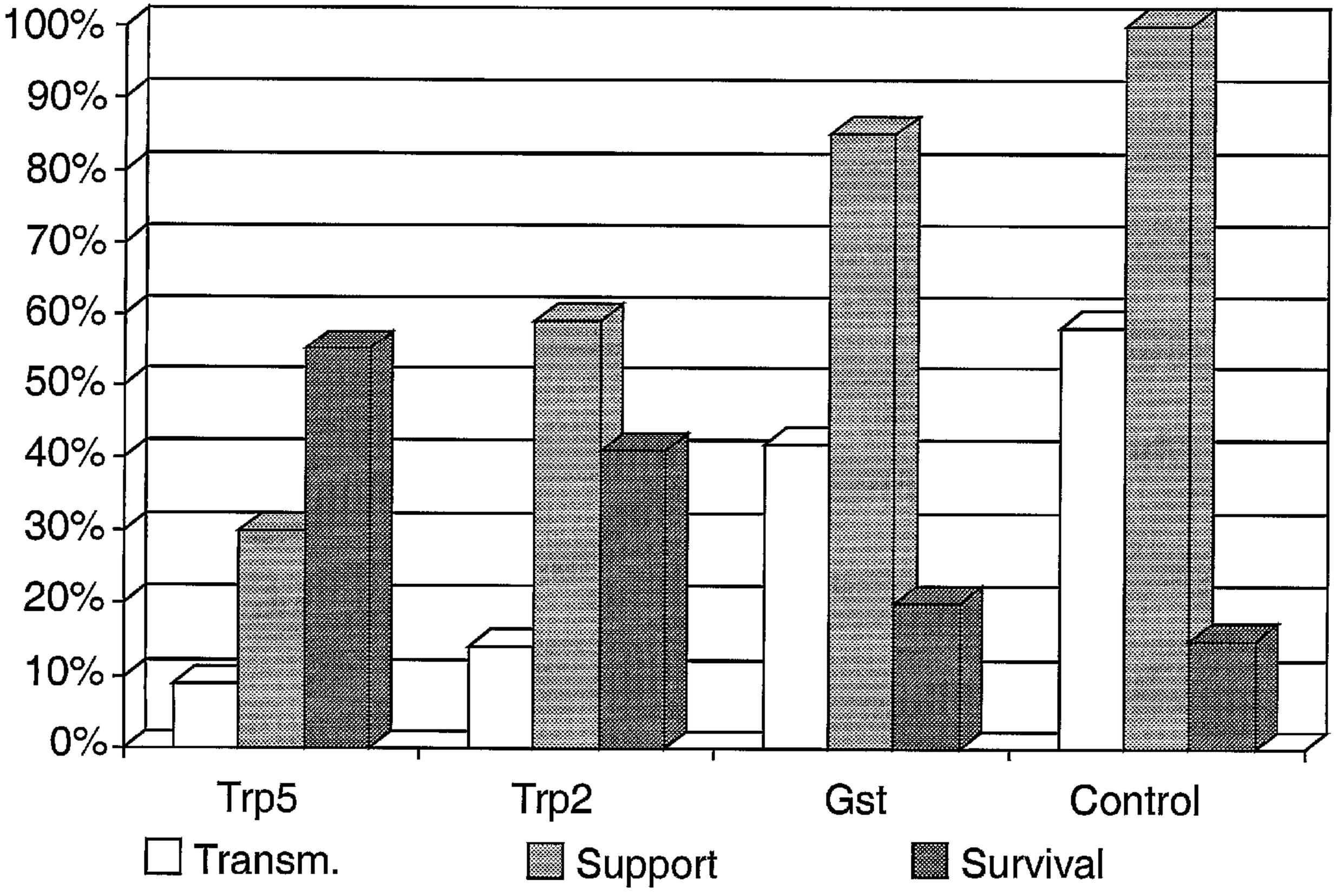


FIG. 8 TBE virus transmission on Balb/c mice, trial I & II

TBE virus transmission on Balb/c mice, trial I & II