The biological activities of [Prot^3]TRH[9]

Compound	Receptor-binding affinity	Releasing activity	
	IC_{50} (nM) (n)	TSH (%) (n)	α-MSH (%)
TRH	32 ± 2.8 (8)	100 ± 9.8[a] (6)	100 ± 5.6
[Prot^3]TRH	49 ± 5.0 (4)	98.7 ± 12.9[a] (6)	104 + 13.6

[a]TSH releasing activity in vivo 20 min after i.p. injection of 5 µg/kg b.wt in female rats. Mean ± SE of n experiments (receptor-binding) or animals (TSH-release).

lowering the TFA temperature to 0 °C, reducing the time of exposure to TFA and immediately coupling with Glp-OTcp. The crude **VI** was chromatographed on silica (70% yield, calculated on the amount of **IV** used) to give a product containing only a trace of a highly polar impurity. The removal of the DNP group from **VI** was accomplished with mercaptoethanol to give the final product **VII**, which could be purified by chromatography on silica and Sephadex G-10 (80% yield). The correct structure of **VII** was proved by ^{1}H-NMR and ^{13}C-NMR spectroscopy, mass spectrometry, amino acid and elemental analyses. The homogeneity of **VII** was demonstrated by TLC and HPLC. HPLC showed it to be completely free from the corresponding D-His diastereoisomer. On the other hand, another synthesis of **VII** from the N-terminal dipeptide[13] and **III**, (2+1 coupling) gave a product containing 21% of the LDL form as estimated with this method.

Stability of [Prot^3]TRH. The possible degradation of **VII** at different pH values was examined by HPLC. The peptide is fully stable in 5% acetic acid. At pH 7.4 about 10% decomposition products were formed in 4 days (1–2% of TRH). In water, the half-life of **VII** was estimated to 8–9 days. All stability tests were performed at room temperature.

Biology. The binding affinity of **VII** for TRH receptors in the membrane fraction of the rat anterior pituitary was determined[14] and the result is presented in the table. The α-MSH-releasing activity was studied in perfused frog neuromediate lobes[15]. The TSH-releasing activity of the analogue in the rat pituitary is also presented.

As seen from these results, there is a good correlation between the three biological tests. [Prot^3]TRH appears to be approximately equipotent with synthetic TRH in vitro and in vivo.

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9 Abbreviations: The symbols for the amino acids and peptides are in accordance with the Recommendations 1983 of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem. *138* (1984) 9). DCC – dicyclohexylcarbodiimide, DNP – 2,4-dinitrophenyl, MA – mixed anhydride with isobutyl chloroformate, OTcp – 2,4,5-trichlorophenyl, TFA – trifluoroacetic acid, HOBt – N-hydroxybenzotriazole, LR – 2,4-bis-(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (Lawesson's Reagent), IC_{50} – the concentration of drug inhibiting specific binding by 50%.
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Babesia bovis: the effect of acute inflammation and isoantibody production in the detection of babesial antigens

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Summary. Immunoblots of *Babesia bovis* antigen contain dominant antigens which react not only with antisera to *B.bovis* but with sera from naive calves recovering from an acute inflammatory reaction. It seems likely these antigens are from the host rather than the parasite.

Key words. Babesia; antigen; inflammation; immuno blotting.

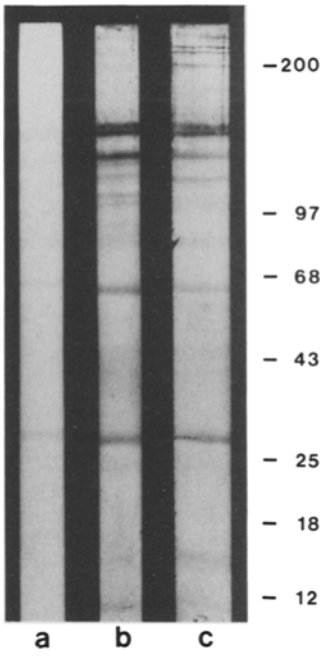
The intra-erythrocytic protozoan parasite *Babesia bovis* causes a severe and often fatal infection in susceptible cattle. However, cattle surviving initial infection are immune from reinfection for at least four years and probably for the remainder of their lifespans[1]. A proven and probably the main mediator of protection is humoral antibody as serum and IgG from immune cattle can protect susceptible cattle from an otherwise fatal challenge[2]. Moreover, protection can be induced by vaccinating susceptible cattle with fractions from disintegrated infected erythrocytes[3]. Studies at this laboratory have centred round a systematic fractionation of infected erythrocytes and subsequent vaccination trials with selected fractions. The final product of such a procedure is likely to be a cocktail vaccine of a few protective antigens. Immunoblotting[4] has been used to target for the latter but results described in this manuscript question the usefulness of this technique and suggest that the major antigen(s) detected by blotting are of host rather than parasite origin.

Methods. Calves, 3–6 months of age, were obtained from areas known to be free of the tick vector, *Boophilus microplus*. On arrival at the laboratory they were tested for *B.bovis* or its

antibodies by thick film analysis[5], and indirect fluorescent antibody (IFA) testing[6]. Calves negative for these tests were then maintained under conditions that precluded accidental tick infestation. Serum samples were obtained from 25 such calves as a pool of negative sera. As well, four calves were randomly selected and an acute inflammatory response induced by turpentine injections. The calves were s.c. injected with 4 ml of turpentine and the injections repeated 3 days later. Prior to the first injection and four days after the second injections, 10 ml of serum was obtained from each calf and stored at −20 °C.

Reference antisera were obtained from field cattle naturally immune to *B. bovis*. Reference *B. bovis* (Samford strain) crude antigen was obtained by mixing equal volumes of the following: 1) a distilled water lysate of infected erythrocytes from which oxyhemoglobin was removed by cation exchange chromatography and 2) an ultracentrifugal supernatant of sonically disrupted parasites and erythrocyte stroma from which residual oxyhemoglobin was removed by preparative gel filtration. Both preparations are used at this laboratory as starting antigens and the hemoglobin fraction of each is devoid of antigen as assayed by hemagglutination (HA) and enzyme linked immunosorbent assay (ELISA)[7,8]. A similar oxy-hemoglobin-free preparation for control purposes was obtained from erythrocytes of uninfected calves.

Sodium dodecyl sulphate (SDS) electrophoresis was performed at 5–10 °C in 80×80×1.5 mm slabs containing a linear gradient of 3–15% acrylamide. The systems of Laemmli[9], Neville[10], and Weber and Osborn[11] were used on different occasions. Prestained protein standards ranging in size from 200 kDa (myosin H chain) to 12 kDa (cytochrome C) were obained commercially[12]. Prior to electrophoresis, samples and standards were made to 1.5% with both SDS and mercapto-ethanol and incubated at 56 °C for 30 min. Following electrophoresis proteins in the gels were transferred to nitrocellulose sheets, both in the presence and absence of methanol[4]. The sheets then were blocked with either 0.25% gelatin, 3% bovine serum albumin or 0.2% Tween 20 in buffered saline pH 7.6 for 1 h at 22 °C followed by 18 h at 4 °C. Antigen bands in the nitrocellulose blots were developed, essentially according to the method of Hawkes et al.[13], by sequential applications, with intermediate washings, of optimally diluted bovine sera, rabbit antibovine IgG coupled to horse radish peroxidase and a substrate solution of 4-chloro-α-naphthol and H_2O_2. If required, bovine sera were firstly absorbed by mixing with an equal volume of control antigens at 37 °C for 1 h.



Immunoblot of *B. bovis* antigen following SDS electrophoresis in 3–15% linear acrylamide slabs. Developed with *a* normal bovine serum. *b* Serum from the same calf but obtained after turpentine injection. *c* Bovine anti-*B. bovis* serum. Relative mobilities and molecular sizes (kDa) of standards are designated at the side.

Results and discussion. Nitrocellulose blots of *B. bovis* reference antigen, after reaction with *B. bovis* antisera showed numerous bands ranging in size from those unable to enter the acrylamide matrix to those migrating near the smallest protein standard. At least 20 antigen preparations were tested over a 3-year period and all produced dominant bands of high molecular weight (> 120 kDa) which were still reactive after absorption of antisera with control antigen and which were not detected in blots of control antigen. However the dominant band varied in size between different antigen preparations. The most common reactions were either at 200 or 160 kDa and occurred in ~ 24% and ~ 36% of the preparations respectively. However in other preparations, the size of the dominant band ranged from 120 to 240 kDa. Nevertheless, the dominant band of each *B. bovis* antigen could be detected, albeit weakly, by approximately three quarters of normal sera and was still detected after prior absorption of both normal sera and antisera with control antigen. Moreover, if the blots of antigen were developed with sera from turpentine treated noninfected calves, the intensity of the main band was markedly enhanced and in addition many of the bands detected by immune sera were also detected. The figure shows blots of one antigen after reaction with a) naive serum, b) serum from the sample animal but following turpentine injection and c) *B. bovis* antiserum. As a generalization, the dominant antigen could be detected by reactive negative sera at a 1:25 dilution, at a 1:125 dilution of sera from calves treated with turpentine and by immune sera at a 1:625 dilution. The initial question arising from the foregoing results is whether the blotting reactions are specific immunologically. This seems so as 1) the reactions did not occur when the secondary antibody marker was used alone, 2) the reactions occurred with Sephadex G200 IgG fractions of the sera tested, 3) the reactions occurred irrespetive of the different buffer systems used during electrophoresis and the different blocking agents used for the nitrocellulose blots,4) nonspecific binding of IgG in primary sera would have given a similar result with all sera irrespective of their origin. Therefore, assuming that the reactions are specific an immediate deduction is that the dominant antigens are not of babesial origin as 1) they were readily detected by sera of naive animals that had suffered an acute inflammatory response and 2) their molecular size variation would be consistent with host genetic variation in isoantigens rather than parasite variation. Alternatively it could be argued that the dominant antigens are of babesial origin and are cross-reacting with a natural isoantibody enhanced by inflammation. Their origin is thus intriguing. Two speculative explanations at present are 1) an increase in erythrocytic isoantigen in infected erythrocytes, as compared to uninfected erythrocytes, to a level detectable by natural isoantibody in serological assays 2) the ability of modified erythrocytes to bind acute phase proteins[14]. *B. bovis*-infected erythrocytes have altered membranes[15,16], and complexing with host acute phase proteins or tissue proteins could cause the latter to react with host isoantibody.

As the height of parasitemia approaches two events occur. One is an acute inflammatory response with a resultant increase in acute phase proteins[17] and the other is an immune suppression and loss of immunological memory induction[18]. Thus at the time when maximum amounts of parasite antigen are being presented to the host's immune system the latter's capacity to induce a primary response to parasite antigens is not only diminished but there is an enhanced secondary response of isoantibody due to acute inflammation. The latter may give credence to Maegraith's

statement[19] that the body reacts in a similar manner to malarial parasites as it would to inflammatory reactions.
A parallel finding to that of immune blotting was observed in a detailed study of IFA for *B. bovis*[6]. The latter conclusively demonstrated that sera of all cattle from tick free areas would stain both parasites and infected erythrocytes but would not stain uninfected erythrocytes. This staining disappeared at dilutions greater than ~ 1:50 in most but in some individuals reactions were obtained at 1:400. Hence a second serological assay complements the immunoblotting results presented here and is additional proof that most normal sera recognize antigens in *B. bovis* infected erythrocytes. The results therefore demonstrate that the antibody response to *B. bovis* antigens is in part paralleled by an increase in host isoantibody. As such the latter may erroneously target for *B. bovis* antigens when immunoblotting techniques are used. The results thus are in accord with a recent finding that many people, including babies, who had no record of parasitic infections, contained antibodies which reacted in immunoblots with major antigens of a spectrum of protozoan and helminthic parasites[20]. The accordance thus raises the question whether decoy antibody and antigen mimicry in parasite infections are more prevalent than suspected.

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Cytotoxicity of human peripheral blood T-lymphocyte clones activated by hepatitis B virus surface antigen

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Summary. The present studies examined the cytotoxic activities of peripheral blood lymphocytes (PBL) from volunteers with (sero-positive) and without (sero-negative) circulating antibodies to hepatitis B virus surface antigen before and 30 days after vaccination with hepatitis B virus surface antigen (HBsAg). Long-term culture of monospecific hepatitis B surface (HBsAg)-responsive T-lymphocytes were isolated and grown in large numbers. The mechanism of T-cell mediated cytolysis, and the identification of the carbohydrate determinants on the surface of these effector cells responsible for the killing effect, are being examined.
Key words. T-lymphocytes; natural killer cells; killer cells; cytotoxicity; hepatitis B virus surface antigen; hepatitis B virus-induced immunity.

Peripheral blood T-lymphocytes regulate various immune responses to most antigens[2,3]. They recognize antigens only in association with the major histocompatibility gene complex encoded molecules (Ia or DR) present on the surface of accessory cells[4,5] known as antigen presenting cells. When triggered by the antigen, the T-lymphocytes proliferate and secrete factors that enhance or suppress the production of immunological effector molecules (antibodies) and/or cells (cytotoxic lymphocytes).
When incubated in vitro, PBL obtained from hepatitis B vaccine recipients respond to HBsAg[6-8]. In presence of the antigen, T-cell growth factor (i.e. Interleukin-2) and Solcoseryl, peripheral blood mononuclear cells from vaccine recipients develop HBsAg-specific T-lymphocyte clones. In response to HBsAg, these clones proliferate, develop into helper T-cells and secrete immunoregulatory factors, interferon and B-cell growth factors[10].
Earlier studies from these laboratories[11] examined the biosynthesis of tumor associated antigen (TAA) in a cell-free system. They demonstrated that glycosylation of the TAA-protein moieties alters the interaction of TAA with its antibodies, and constitutes a post-translation modification of gene expression. The availability of purified glycosidases which permit the stepwise removal of each carbohydrate component[12,13] have prompted the use of HBsAg-specific T-lymphocyte clones as a model system in studying the characteristics of the cell-surface glycoproteins in effector-target cell interactions.
Materials and methods. Heptovax® (Merck Sharp Dohme, Product no. 38676) highly purified, sterile filtered solution 40 µg/ml. Vibrio Cholera neuraminidase (VCN), *E. coli* β-galactosidase (β-Galase), almond fucosidase (Fucase) and endo-β-N-acetylglucosaminidase were purchased from Sigma Chemical Co. Absence of proteinase activity in these glycohydrolase preparations was determined colorimetrically after incubation of 0.1 unit of either enzyme with each of the following substrates: Azocoll, casein yellow, denatured hemoglobin, and azocasein in 1.0 ml of 0.5 M NaAc, pH 5.5 at 37 °C for 24 h.
Solcoseryl (Solco, Basel, Switzerland), a deproteinized extract of calf blood containing 45 mg dry substance of which approximately 70% consists of inorganic salts, and the remainder contains amino acids, hydroxy and keto acids, deoxyribose, purines and acid and alkaline polypeptides[14] was used at 1% as a supplement in the culture media.