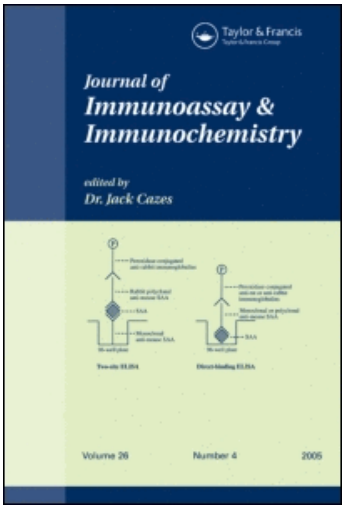

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RADIOIMMUNOASSAY FOR BOVINE TUMOR NECROSIS FACTOR: CONCENTRATIONS AND CIRCULATING MOLECULAR FORMS IN BOVINE PLASMA

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ABSTRACT

Antisera against recombinant bovine tumor necrosis factor (rbTNF) were produced in rabbits immunized with rbTNF in Freund's complete adjuvant (F314) and used in a double antibody radioimmunoassay to measure plasma TNF. Assay standards were rbTNF. Iodination of rbTNF and chromatography on G-50 Sephadex with 50 mM EDTA, 0.1 % BSA, 0.05 M phosphate buffer, pH 7.5 resulted in labelled rbTNF which was >97% TCA precipitable (specific activity of 37.5 μCi/μg). F314 (1:80,000 dilution) bound 21% of ^{125}I- rbTNF in a non-equilibrium assay at 4 C. Separation of bound and free ^{125}I-rbTNF was accomplished by precipitation with goat anti-rabbit IgG prepared with 6% polyethyleneglycol (mw = 8000). Minimum detectable TNF was 4 pg/assay tube. Matrix effects of plasma were minimal. Recovery of rbTNF from plasma was linearly (recovered TNF = .932 * added TNF - .12; r = .99). Displacement curves of increasing amounts of plasma from calves challenged with endotoxin to effect an increase in endogenous TNF were parallel to the rbTNF standard curve. F314 failed to crossreact with any other cytokines tested except human TNF (<1%). Neither recombinant nor native bovine TNF significantly interacted with antisera for TNF of human or murine origin. Plasma TNF was acutely elevated in calves infused with endotoxin. Changes in plasma TNF were determined in samples from calves with chronic parasitic infection. Endogenous plasma TNF existed as a monomer with a molecular weight of 17,000, and was not bound to any plasma carrier protein. These data indicate that a specific RIA for bTNF capable of detecting changes in in vivo TNF levels has been established.

(KEY WORDS: Tumor Necrosis Factor, Radioimmunoassay, Cytokines)

INTRODUCTION

The cytokine tumor necrosis factor-alpha (TNF), also known as cachectin, is secreted by macrophages in response to various pathogens (1). The existence of this 17,000 MW protein was first demonstrated by Carswell et al. (2) as a macrophage factor, inducible by bacterial lipopolysaccharide administration, which caused hemorraghic necrosis of certain tumors in mice. Work by Palladino et al. (3) demonstrated the ability of TNF to kill several transformed cell lines. An independent line of research implicated a peptide factor, which had been named cachectin, and was ultimately shown to be identical to TNF, as the mediator of cachexia and shock in certain disease and infectious states (4). Included among the diverse biological activities of this compound were the enhancement of fibroblast growth (5), stimulation of bone resorption (6), and inhibition of a number of lipogenic enzymes and associated proteins (7). Because of these and other reports, TNF has been proposed as a mediator of the metabolic disturbances observed in acute infections, such as with endotoxemia, and in chronic infections such as malaria (8,9), bacterial infections (11) and parasite infections such as Sarcocystis (12).

Until recently, the cytotoxicity bioassay has been used as the definitive test for TNF activity. Although the assay is apparently sensitive to TNF across species, it has limitations that preclude rapid determination of TNF levels in large numbers of samples. Development of a radioimmunoassay (RIA) for bovine TNF would permit a more accurate and rapid quantitation of TNF, thereby permitting deeper investigation of the role of TNF in mediating hormonal and metabolic changes, tissue wasting and stunted growth observed in acute and chronic infections in cattle. Therefore, the objectives of these studies were to develop and validate a

RIA for TNF which would specifically determine TNF concentrations in plasma. In addition, the assay would be used to quantitate plasma TNF in 1) calves which had been challenged with endotoxin and were acutely ill, and 2) calves which were experimentally infected with Sarcocystis cruzi, as models for investigation of the role of TNF in acute and chronic infection. The final objective was to characterize endogenous TNF activity in plasma.

MATERIALS AND METHODS

Development of antisera

Two New Zealand white female rabbits were injected intradermally at 0 and 14 d with 15 μg recombinant bovine TNF (rbTNF) in 1 ml of either RiBi adjuvant (muramyl dipeptide, RiBi Immunochem Research) or Freund's complete adjuvant diluted 1:1 with sterile saline in 16-20 sites randomly distributed over the dorsal surface from the scapula to pelvis . Recombinant bovine TNF (6.4 X 10 E05 U/mg by bioassay; 0.16 U/mg endotoxin by limulus amoebocyte lysis assay; >99% pure) was generously donated by Dr. Serge Martinod (CIBA-GEIGY Limited, St. Aubin-FR, Switzerland). Titer to TNF, determined as dilution of serum which bound 50% of added 125-I rbTNF, was monitored in blood drawn rabbits under ketamine-acepromazine analgesia from the ear vein on d 28, 35, 42, 49 and 56 for rabbit initially injected with Freund's adjuvant and on d 28, 35, 42 for RiBi adjuvant rabbit. Because antibody development to the recombinant bovine tumor necrosis factor was minimal using either adjuvant regimen, it was decided that an additional change in the immunological presentation of the molecule needed to be introduced to improve antibody elicitation. Both rabbits were boosted intradermally at 89 d with 1 ml Freund's complete adjuvant containing 62.5 μg of rbTNF conjugated to itself to form a polymerized TNF. Conjugate was prepared by combining 250 μg rbTNF in

2 ml 0.1 M ammonium acetate buffer (pH 7.0) and 1.3 ml 0.02 M glutaraldehyde and allowing mixture to react for 4 h at 23 C with subsequent dialysis and lyophilization. Titer was measured as before in blood drawn on d 89, 106, 117, 131, 189 and 210. Both animals were boosted a second time on d 210 with 15 μg TNF conjugate in 1 ml Freund's incomplete adjuvant. Blood was sampled on d 214 and 221 and titer determined.

Iodination procedure

Recombinant bovine TNF was iodinated using minor modifications of the iodogen method described in Elsasser et al. (12). Radiolabelled TNF was produced through addition of 50 μl of 0.5 M sodium phosphate buffer (pH 7.5) to a septum-stoppered glass vial precoated with 2 μg iodogen (Pierce Chemical). The vial was gently agitated for 12 min after addition of 0.5 μCi $Na^{125}I$ (New England Nuclear) and 11.5 μg TNF. Iodinated protein was purified by anion exchange chromatography (Biorad, AG 2 X 8) and gel permeation chromatography on 1 X 30 cm Sephadex G-50 (20-50 micron) using assay buffer (0.05 M phosphate-buffered saline (0.135 M NaCL), 50 mM disodiumethylenediaminetetraacetic acid (EDTA, Aldrich), 0.1 % BSA, 0.1 % between 20, 0.01 % thimerosal; pH 7.4) as elution buffer. Iodinated TNF had a specific activity of 37.5 μCi/μg as determined by the self displacement technique described by Jaffe (13). Unless specifically noted, all chemicals and biologicals were obtained from Sigma Biologicals.

Assay procedure

The assay was performed as either an equilibrium or nonequilibrium (with delayed trace addition) assay. Volumes of reagents for either assay method were the same; assays differed only in respect to the time of tracer addition (simultaneous addition with specific anti-TNF antisera in

equilibrium assays and trace added after 24 h preincubation at 4 C of unknowns or standards and antibody in nonequilibrium assay) and length of primary incubation (24 h at 4 C for equilibrium, 18 h for nonequilibrium). Anti-TNF (first antibody) was added in 100 μl volume in working concentration of 1:20,000; diluted in assay buffer without BSA (-BSA buffer). Standards (serial dilution of 1.25 ng/tube to 9.8 pg/tube TNF in assay buffer in nonequilibrium assay and 2.5 ng/ tube to 39 pg/tube in equilibrium assay) and unknowns routinely added in 100 μl volume, but range of 6.75 to 200 μl volume for unknowns tested in recovery and parallelism studies. Radiolabelled TNF was diluted in assay buffer to 10,000 CPM/100 μl and added to all tubes last. Final volume in total binding (0 standard), non specific binding (no first antibody) and standards/unknowns was 400 μl. Specific hormone-antibody complexes were precipitated by addition of 400 μl of 1:60 goat anti-rabbit IgG (Research Products International) and 6 % 8000 MW polyethylene glycol mixed in a 2:5 ratio, to all tubes (except total counts) and were incubated at 4 C for 1 h. Tubes were further incubated at 4 C for 30 min after addition of 100 μl 1% normal rabbit serum in buffer and then centrifuged at 1850 x g for 20 min at 4 C, aspirated and counted.

Assay validation

The radioimmunoassay developed for bovine TNF-alpha was validated through evaluation of quantitative recovery of added hormone, parallelism of plasma with high levels of native (endotoxin stimulated) TNF, and crossreactivity of the antisera with other hormones and cytokines. Using an equilibrium assay, recovery of recombinant bovine TNF was estimated by comparing the measured amount of TNF in a given volume of plasma to the mass of rb TNF added to the plasma after subtracting the mass of TNF endogenous to the plasma. Over a series of added quantities of TNF (125,

250, 500, 750, 1000 and 1250 pg/200 μl plasma), a regression analysis of added TNF versus recovered TNF mass was used to determine the efficiency of recovery (slope of the regression equation:y(recovered) = mx(added) + b, where m is the slope and b is the intercept). The ability of the antisera against TNF to equally recognize native TNF and recombinant TNF was evaluated through comparison of the ability of increasing amounts of added recombinant TNF and increasing volumes of plasma from endotoxin-stimulated calves to displace labelled TNF. Crossreactivity of d 221 serum from rabbit initially injected with Freund's complete adjuvant (designated F314) with 1000, 250, 62.5, 15.6, 3.9, 0.975, 0.244 and 0.061 ng/tube of bovine GH, recombinant bovine interleukin - 2 (IL-2), recombinant human gamma Interferon (IFN-beta), human TNF-alpha and human TNF-beta was evaluated in an equilibrium assay. Recombinant bovine IL-2 was generously donated by Dr. Nancy Magnusen, Washington State University and recombinant human gamma INF-beta, TNF-alpha and TNF-beta were donated by Genentech, Inc. S. San Francisco, CA.

Measurement of TNF activity in plasma

TNF concentrations were measured by RIA in plasma of four Holstein bull calves (100 kg) which were challenged with intravenous doses (1 μg/kg) of E. Coli serotype 055:B5 endotoxin (Sigma Chemical, St. Louis, MO) in 5 ml of sterile saline and saline alone (control). Sterile solutions were administered via indwelling teflon (Abocath 14) jugular catheters. Blood samples (7 ml) were collected into tubes containing 15 mg EDTA at -2, -1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h relative to injection (t = 0) and were kept in an ice bath for no longer than 4 h before centrifugation at 1850 g and the plasma pipetted off and frozen at -20 C. Plasma TNF in three Holstein bull calves (65 kg initial weight)

experimentally infected with 300,000 sporocysts of Sarcocystis cruzi and three noninfected controls was evaluated by RIA at 0, 14, 21, 26, 28, 30, 35, 44, 49, 56, 63, 70, 77, and 84 d postinfection. Sarcocystis cruzi is a protozoan parasite which undergoes two of its three life-cycle stages in the bovine and can cause severe stunting of growth and tissue wasting in infected growing calves, and abortion and infertility in cows (10,11). Blood samples were drawn by venipuncture into tubes containing EDTA as the anticoagulant and processed as described above.

Characterization of plasma TNF activity

Endogenous plasma TNF immunoactivity was characterized using molecular sizing over a 1 X 30 cm Sephadex G-50 column equilibrated in assay buffer at 10 C. Plasma samples corresponding to the highest TNF concentrations measured following the endotoxin administration described above were pooled. The TNF activity in the fractions (0.45 ml) resulting from G-50 separation (using assay buffer as eluting buffer) was determined by RIA. To investigate the possibility that native TNF might circulate within the bloodstream bound to either a specific or nonspecific carrier protein, another 200 ul of the pooled plasma was incubated for 24 h at 4 C with 125-I rbTNF (2 X 10 9 CPM in 100 ul assay buffer). The plasma and trace mixture was then chromatographed on the Sephadex G-50 column exactly as described for plasma alone, and the fractions counted on a Packard 3000 Gamma counter (Packard Instrument Company, Inc., Downers Grove, IL). These two elution profiles (plasma and plasma plus trace) were compared with elution of 125-I TNF alone from the G-50 column under the same conditions.

RESULTS AND DISCUSSION

Titer measurements in sera from both rabbits immunized against rbTNF are displayed in Figure 1. Although not discernible because of scale,

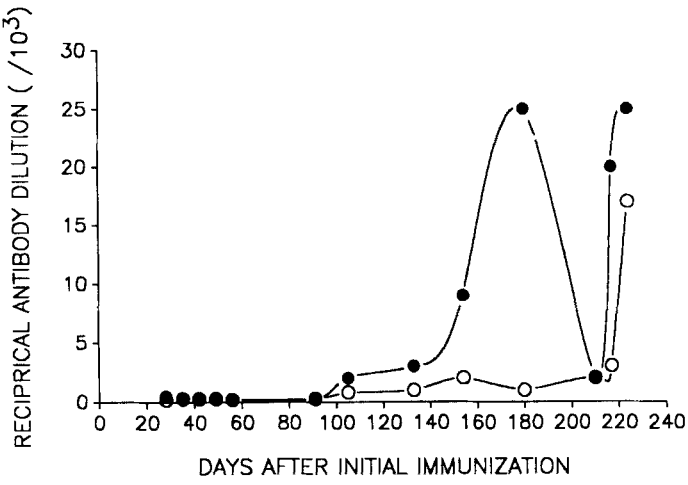

FIGURE 1. Development of serum antibody titer in rabbits injected with recombinant bovine tumor nectosis factor in either Freund's adjuvant (●) or RiBi adjuvant (○) containing muramyl dipeptide and boosted with antigen at 85 and 210 days. Titer based on precipitation of 50% of available ^{125}I-rbTNF counts with goat anti-rabbit gamma globulin in 6% polyethyleneglycol 6000.

after the initial immunization, titer against TNF increased to a peak of 1:210 at 42 d for the rabbit receiving TNF-Freund's adjuvant mixture and to 1:80 at 28 d for the rabbit initially receiving the TNF-RiBi adjuvant mixture. Antibody production against TNF was progressively stimulated with each successive booster injection and increased from 1:210 and 1:80 after the initial immunization to 1:520 and 1:2000. However, even with a dilution of 1:2000 the utility of the preparation for use in a radioimmunoassay was questioned. The poor titer development was considered to be due to a possible species similarity between bovine and rabbit TNF such that the immunogenicity of the recombinant TNF was low. Having had success improving the antibody response to other peptide

hormones by effectively crosslinking the peptide to itself with glutaraldehyde, the recombinant TNF was subjected to similar treatment. Relative success of this procedure was evident in the significant increase in titer 21 d after the boosting with the coupled material (Figure 1). Titer increased dramatically to 1:26,000 at d 175 in the Ribi adjuvant rabbit; however, testing of this serum (data not shown) indicated that it was not appropriate for use in the assay as it did not yield a suitable standard curve based on minimal sensitivity, stability and freedom from matrix effects. Although serum from both rabbits contained high titer to TNF on d 221 (Figure 1), serum taken from the rabbit which received Freund's adjuvant initially, was selected for development of the RIA because it yielded a superior standard curve in terms of assay sensitivity (Table 1.)

As shown in Figure 2, iodination of rbTNF and characterization by Sephadex G-50 gel chromatography was successful in the generation of a labelled hormone fraction of uniform molecular mass. With acidification of an aliquot of the tracer by 10% trichloroacetic acid, 97% of the radioactivity was precipitated in the protein. In addition, 95% of the radioactivity was bound by an excess of F314 (1:500) rabbit anti-TNF. In the Figure, only radioactive counts associated with iodinated peptide are present due to prior adsorption of free radioiodine with an anion exchange resin.

An assumption made in the validation of radioimmunoassay is that there is no difference in the immunorecognizability of the native antigen and the iodinated antigen by the antiserum. As depicted in Figure 3, the slopes of the displacement curves for standards of rbTNF and increasing volumes of iodinated rbTNF tracer were parallel. Using the slope as an indication of the affinity of the antigen for the antiserum, it is evident

Table 1

Comparison of Assay Parameters From Standard Curves Generated From Rabbit Anti-bovine TNF Serum Collected 221 Days Postimmunization of Rabbits Challenged With Antigen In Either Freund's or Ribi Adjuvants.

Assay Parameter	Freund's Adjuvant	Ribi Adjuvant
Serum Final Dilution	1:100,000	1:120, 000
Tracer Binding (%)	32.0	28.2
Slope[a]	-2.53	-2.47
B_{50} (ng/tube)	0.091	0.189
r^2	0.98	0.99

[a]Regression of logit (B/B_0) vs $\log_{10}$mass (ng) rbTNF added/tube; B=specific binding at each rbTNF mass level; B_{50} is the mass of rbTNF that effectively displaces 50 percent of the bound tracer counts

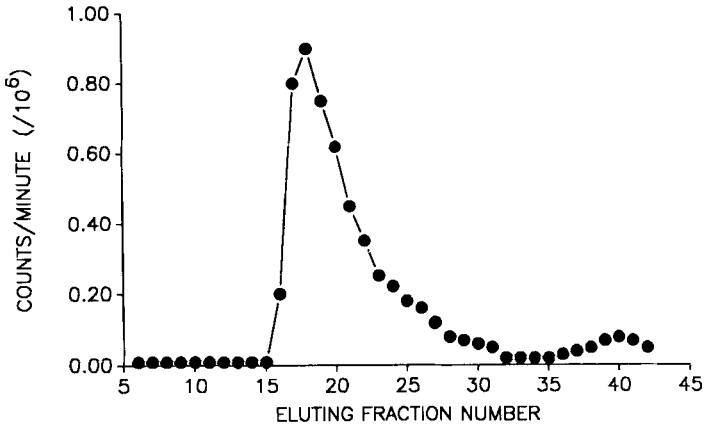

FIGURE 2. Elution profile of recombinant bovine tumor necrosis factor following iodination and separation on Sephadex G-50 (20-50 μ). Free iodine peak not present due to prior adsorption of iodination mixture on an anion exchange resin bed. Data indicate uniformity of molecular species and absence of aggregation.

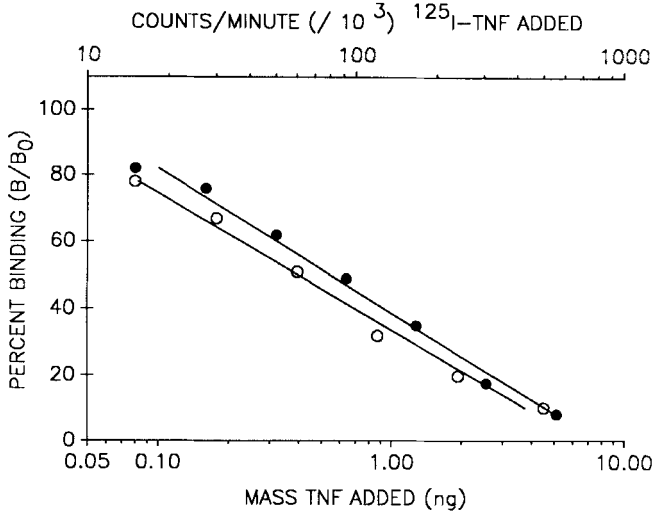

FIGURE 3. Linear displacement of tracer by nonlabeled recombinant bovine tumor necrosis factor (●) and parallel displacement of counts with increasing volumes of iodinated tracer (○) for the determination of specific activity by the method of self-displacement and evidence of similar recognition of natural and iodinated forms of the peptide.

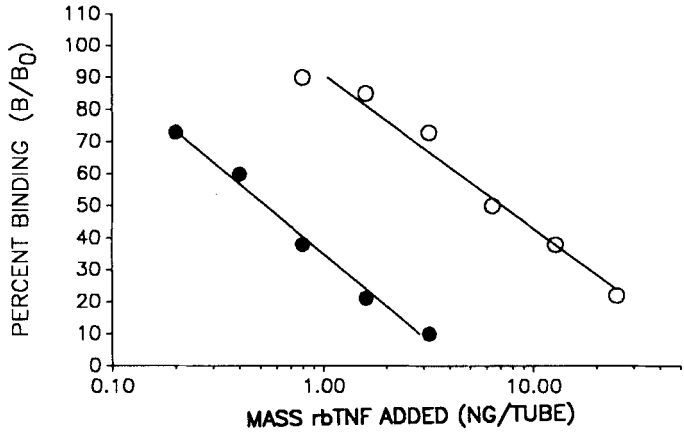

FIGURE 4. Differences in assay sensitivity as effected by incubation of tracer with antisera under equilibrium (●) and nonequilibrium (○) conditions.

that iodination of rbTNF did not alter its recognition by the antisera. In addition, data in this figure demonstrate that iodination of rbTNF by the method cited resulted in the generation of labelled hormone with a specific activity of 37.5 μCi/μg, as calculated by the method of self-displacement.

Delayed trace addition and a decreased primary incubation period in the nonequilibrium assay was found to profoundly increase assay sensitivity as compared to the equilibrium assay (Figure 4). The minimum detectable quantity was decreased by a factor of 10 (from 40 pg to 4 pg), while the B_{50} dose (mass rbTNF added which displaced 50 % of trace) was depressed 15 fold (.7913 ng to 0.0484 ng) in simultaneous assays performed at a final first antibody dilution of 1:100,000. In this comparison, total binding was not deleteriously affected by nonequilibrium conditions (17 vs 21% for nonequilibrium and equilibrium, respectively), although with other assays and tracer lots, it has been found to occur to a minor degree (depression of 3-5 percentage units). The slope of the regression of logit B/Bo vs $\log_{(10)}$ mass added, was higher ($P<.05$; Student's T-test) in this comparison, additionally indicating that the nonequilibrium protocol resulted in binding kinetics that favored a more sensitive assay. Therefore, in experimental situations where pronounced elevations in TNF levels are expected or are possible, such as with endotoxemia, an equilibrium assay is recommended, while plasma TNF concentrations in chronic or subclinical infections should probably be evaluated under nonequilibrium conditions.

Recovery of rbTNF added to a standard pool of bovine plasma, under nonequilibrium assay conditions, is depicted in Figure 5. Recovery of TNF was assessed by regression analysis. The relationship between the known quantity of rbTNF added to plasma versus the mass of rbTNF measured

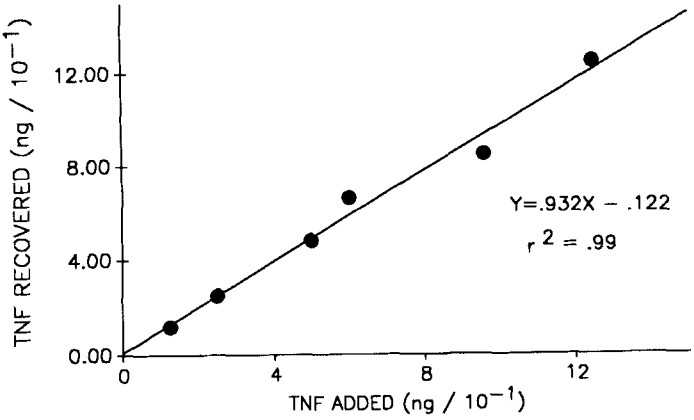

FIGURE 5. Recovery of nonlabeled recombinant bovine tumor necrosis factor added to bovine plasma. To a fixed volume of bovine plasma (200 μl) increasing mass of recombinant bovine TNF (125-1250 pg/tube) are added under equilibrium conditions. The recovery of the nonlabeled peptide is represented by statistical regression analysis across the known masses of TNF, added relative to the mass measured in the assay and numerically expressed as the slope of the regression equation (equal to the overall percent recovery).

following subtraction of the TNF endogenous to the plasma sample was compared. The resulting linear relationship indicated that the assay could account for roughly 93% of added TNF. It was concluded that the assay accurately predicted TNF concentrations, with little or no interference from plasma.

Data depicted in Figure 6 illustrate that parallel displacement of labelled recombinant TNF was achieved in an equilibrium assay (F314 serum at final dilution of 1:80,000; 17 % binding and intrassay coefficient of variation of 13%), with increasing volumes of plasma from Holstein bull calves challenged with intravenous doses of endotoxin. The slopes of regression lines of logit B/Bo vs log μl plasma for the five high (>10 ng/ml) native TNF plasma samples tested were -1.997, -1.959, -2.037,

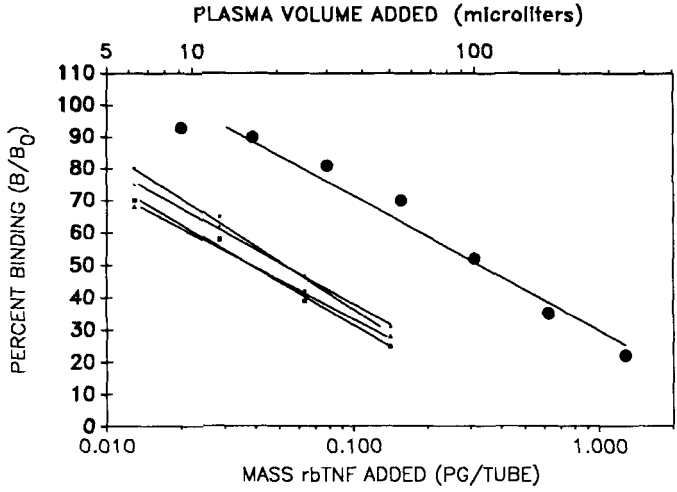

FIGURE 6. Parallel displacement of tracer counts by increasing concentrations of standard recombinant bovine tumor necrosis factor (●) and increasing μl aliquots of bovine plasma from calves injected intravenously with endotoxin (5 μg/kg, n=4, ◆ ◇ ■ □) to effect an increase in circulating endogenous plasma concentrations of tumor necrosis factor.

-2.029 and -2.052, for the samples from animals 472, 474, 478 at 1 h post endotoxin injection and 478, and 472 at 2 h postinjection, respectively. These values were not different from that of the recombinant standard (-1.998), suggesting that native and recombinant TNF were equally competing for binding sites on available anti-TNF. It was concluded that the assay could accurately measure endogenous plasma TNF levels.

Of all the cytokines and hormones tested in the crossreactivity studies performed in the development of this assay, only human TNF-alpha demonstrated any ability to displace labelled bovine TNF (Figure 7). Extrapolation of the hTNF-alpha curve to 50% displacement and comparison with the level of rbTNF required for 50% displacement in this assay would yield an extent of crossreactivity estimate which is <1%. This result was quite surprising in view of the sequence homology which has been

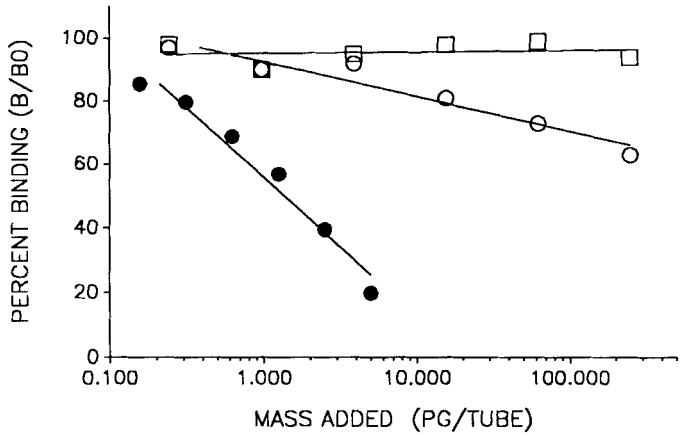

FIGURE 7. Crossreactivity of antiserum against recombinant bovine tumor necrosis factor (● ●), recombinant human tumor necrosis factor alpha (○ ○), and tumor necrosis factor beta, interleukin-2, gamma interferon, bovine growth hormone and bovine insulin (□). All the nondisplacing peptides (95-105 percent binding, □) are represented by a common symbol but each point represents the determination of three replicates of each peptide at each mass.

demonstrated to occur between human and bovine TNF-alpha (14). These data led to the conclusion that the antisera which has been developed for this assay, in spite of the high degree of structural similarity (80%) between human and bovine TNF, was able to specifically distinguish native and recombinant bovine TNF from human TNF, and from other cytokines and hormones as well.

Jugular infusion of endotoxin in Holstein bull calves resulted in an immediate increase in circulating TNF concentrations, (Figure 8). Within 1 h of infusion, TNF levels had increased almost 100-fold from baseline concentrations of approximately 137 pg/ml to 13 ng/ml. Values actually peaked at 2 h postdose. These levels then declined to nonstimulated baseline concentrations by 10 h. Endotoxin administration is known to be a classical inducer of TNF secretion, and this phenomenon has been shown to occur in a variety of species, most notably the mouse (2,15). In fact, there is good evidence that TNF mediates the pathophysiological effects

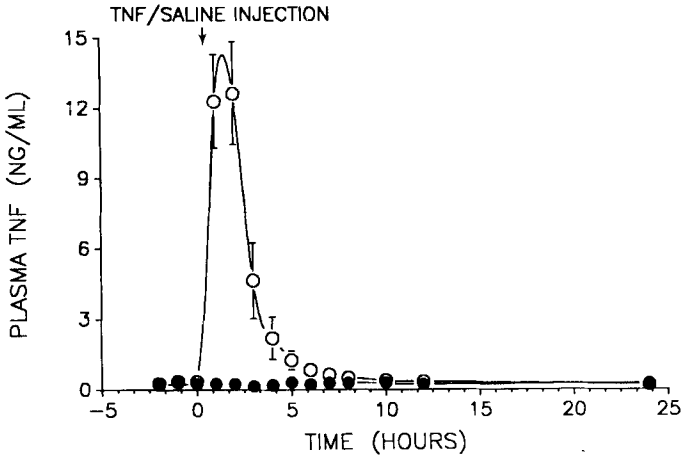

FIGURE 8. Changes in plasma concentrations of bovine tumor necrosis factor in calves following intravenous administration of endotoxin (○) or saline (●). Values represent mean determinations for three calves challenged with endotoxin and four calves that received saline as a control injection.

observed in mice injected with bacterial lipopolysaccharide (16). This study is the first to demonstrate that endotoxin can induce endogenous in vivo TNF secretion in the bovine. Hormonal and metabolic perturbations which have been shown to occur in acute endotoxemia in the bovine, also seem to parallel responses to recombinant TNF administration in calves (17). It is well documented that interleukin-1 is also released following endotoxin challenge in experimental animals (18). The likelihood that any of the measured increase in immunoreactive TNF in bovine plasma was due to crossreactivity with interleukins is small considering the lack of homology between TNF-alpha and interleukin-1 (<20 percent, 19).

Plasma TNF levels in Holstein bull calves experimentally infected with Sarcocystis cruzi are portrayed in Figure 9. In contrast to levels observed in acute endotoxemia, circulating TNF concentrations were not profoundly affected in chronic infection with sarcocystis, although a tendency existed in the 32-84 d period following infection. TNF

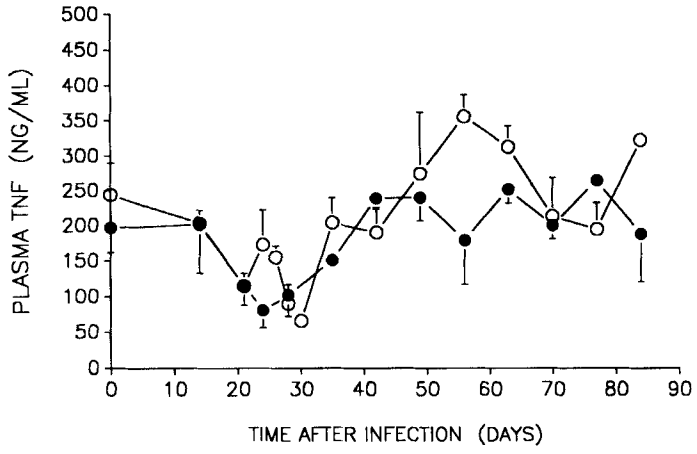

FIGURE 9. Changes in circulating plasma concentrations of bovine tumor necrosis factor in calves inoculated and infected with Sarcocystis cruzi (○) and values for contemporary noninfected calves (●). The shaded area corresponds to the time in the development of the infection during which a significant rise in body temperature and expression of clinical signs are observed in the infected animals. Values represent means for each group (n=4) at that sampling time ± standard error of the mean.

concentrations declined in both control and infected groups during the 28 -32 crisis period when the parasite undergoes its second schizogonous replication, which is associated with elevated body temperature, inappetence and a number of other metabolic and hormonal perturbations (12, 20). The depression in TNF levels in infected calves tended to be greater in this acute period. At this point, the data available do not support a simple relationship between TNF secretion (presumably evidenced by plasma levels) and infection. Further work in this laboratory is continuing to investigate the relationship between circulating and tissue TNF levels in the time course of infection by the parasite. Nonetheless, the assay was successful in detection of relatively small changes in already low (100 -350 pg/ml) circulating concentrations. The plasma concentrations reported here are in line with values reported for other species using ELISA-based analytical techniques (8,21,22). In addition,

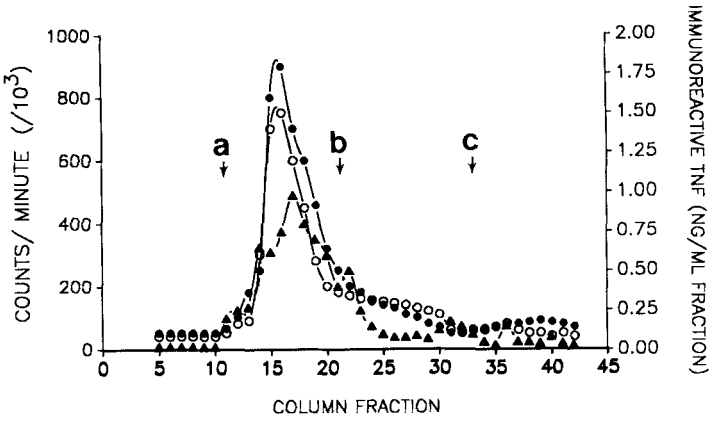

FIGURE 10. Characterization of molecular weight of iodinated recombinant bovine tumor necrosis factor in assay buffer (▲), the same tracer added to normal bovine plasma (○) and as measured in endogenous bovine plasma (●). Molecular weight was determined by the position of elution relative to that observed using carbonic anhydrase (a, 29,000 mw), cytochrome-C (b, 12,400 mw) and iodinated monomeric insulin (c, 6700 mw) as molecular weight standards.

results by other researchers suggest that the relationships between infection type (bacterial, viral, etc.) and the systemic production of TNF is highly variable (23).

Results of experiments which investigated the characteristics of endogenous plasma TNF activity have been combined and depicted in Figure 10. The combined elution pattern data of 125-I TNF alone, pooled plasma from endotoxic calves, and plasma incubated with trace, indicate that endogenous TNF activity elutes in much the same manner as the iodinated recombinant TNF. Peak radioimmunoassayable TNF activity, calculated as 2(Bo/B -1), where Bo and B refer to total binding (no added fraction) and the specific binding of trace by each fraction, respectively, and CPM/fraction recovered from G-50 separation of trace alone and trace incubated with plasma, coincided exactly at fraction 16 (7.2 ml). Since it is known that recombinant TNF standard used for iodination has a molecular weight of 17,000, it was determined that

endogenous immunoreactive TNF activity in the bovine likewise has a molecular weight of 17,000, and exists in circulation primarily as a monomer and is not specifically bound to any carrier protein. These results may be at variance with those described in reports investigating TNF of other species. Recombinant or human TNF activity purified from tissue culture supernatants of serum under denaturing conditions has been reported to consist of a polypeptide of 17,000 or 17,500 MW (24, 25). However, crystal X-ray diffraction studies have indicated that recombinant human TNF consists of a simple trimer (26). Smith and Baglioni (27) reported that the bioactive form of human and murine TNF was also a trimer. However using a sensitive radioreceptor assay for TNF, we have found that binding of multimeric forms of TNF to pituitary membranes to be significantly reduced compared to binding of true monomeric TNF (28). In addition none of the other cytokines, including interleukin-1 and TNF-beta (lymphotoxin) competed with recombinant TNF for membrane binding. Further work is necessary in the bovine to characterize circulating TNF activity in various pathological conditions and to evaluate bioactivity of endogenous TNF secreted in response to endotoxin.

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