Journal of Chromatography, 583 (1992) 11–18
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6554

Immunomagnetic separation of tumor necrosis factor α

I. Batch procedure for human temporomandibular fluid

Edward F. Rossomando, Lorraine B. White and Jane Hadjimichael

Department of BioStructure and Function, University of Connecticut Health Center, Farmington, CT 06030 (USA)

David Shafer

Department of Oral and Maxillofacial Surgery, University of Connecticut Health Center, Farmington, CT 06030 (USA)

(First received April 14th, 1992; revised manuscript received August 20th, 1992)

ABSTRACT

A batch separation procedure has been developed for retrieval of tumor necrosis factor (TNF) α from the microliter volumes of fluid isolated from the human temporomandibular joint (TMJ). Paramagnetic beads coated with monoclonal antibodies for TNF were used. The beads, and bound TNF, were recovered from solution with the aid of a magnetic field. The amount of bead-bound TNF was quantified using an immuno-based assay developed in this laboratory called the cluster assay. The cluster assay was specific for TNF and linear up to 10 ng. Using these methods we found that TMJ fluid contained 0.2–4.2 ng per 100 μl of fluid with a mean value of 1.9 ng and a standard deviation of 1.1 ng. This study demonstrates the utility of batch immunomagnetic separation for the concentration and purification of proteins, and the cluster assay for quantification of proteins from microliter volumes of body fluids.

INTRODUCTION

Separations need not be performed on a column. An alternative, batch separation, is normally performed by the addition of a slurry of a solid phase directly to the solution containing the ligand (see ref. 1 for brief review). A batch separation may be useful when the ligand is present in low amounts and when only microliter volumes of fluid are available. In the present study we used a batch procedure to isolate tumor necrosis factor (TNF) α, a trimeric protein with a molecular mass of approximately 17 000 daltons per monomer secreted by monocytes and macrophages. The biochemistry and biological effects of TNF have recently been reviewed [2]. Previous work from this laboratory has identified TNF in a variety of fluids [3–5] and results from other laboratories have identified TNF in serum [6], synovial exudates from rheumatoid joints [7] and serum of children with severe infectious purpura [8].

The temporomandibular joint (TMJ), the articulation between the base of the skull and the mandible or lower jaw, is lined with a synovial membrane and contains only microliter volumes of synovial fluid [9]. Previous studies have identified inflammatory mediators such as the plasma proteins immunoglobulin G (IgG) and immunoglobulin M [10] and components of the arachi-

Correspondence to: Edward F. Rossomando, Department of BioStructure and Function, University of Connecticut Health Center, Farmington, CT 06030, USA.

0378-4347/92/$05.00

donic acid cascade, including prostaglandin E_2 and leukotriene B_4 [11] in the fluid collected from this joint. While the results of all these studies indicate changes in the biochemical composition in the dysfunctional joint, the cause for the pain and destruction associated with TMJ dysfunction remains unclear. Although some have argued that the pain and dysfunction are a result of muscular hyperactivity and joint disease [9,12,13], others have suggested an aseptic inflammatory reaction in the masticatory muscles [14]. The presence of inflammatory markers [10,11] together with the favorable results obtained with corticosteroids [15] supports inflammation as an explanation. In addition, as TNF induces the secretion of collagenase by fibroblasts [16,17] and has been implicated in the destruction of connective tissue and the resorption of bone [18], isolating TNF from TMJ fluid could provide a mechanism to explain the pain and dysfunction associated with the disease of this joint.

For the separation of TNF-α, we used as solid phase paramagnetic beads precoated with anti-TNF monoclonal antibodies (MAbs). The addition of a slurry of these beads to a microliter sample of the joint fluid resulted in binding of the TNF to the antibody-coated beads. The application of a magnetic field to the solution separated the beads and the attached TNF from the solution, resulting in a concentration and purification of the small amounts of TNF in the fluid. Previous studies have used antibody-coated magnetic beads for the recovery of virus [19], bacteria [20] and cells [21–23] from tissue cultures. In addition, immunomagnetic beads were used for the separation of cells with open column chromatography [24,25].

The quantification of the bead-bound TNF was accomplished using an immuno-based assay developed in this laboratory, called the cluster assay. The method replaces the color-generating system of an enzyme-linked immunosorbent assay (ELISA) with a second bead having a diameter one half that of the bead used for capture. Coating the second bead with a polyclonal, or alternatively, a different epitope seeking MAb allows this smaller bead to bind to unused epitopes on the bound TNF. Thus, the presence of TNF acts as a bridge resulting in the formation of a large bead–small bead complex or cluster. On the basis of the number of clusters, a suitable calibration curve can be constructed allowing the quantification of the amount of TNF present in the fluid. Using the cluster assay and immunomagnetic separation, here we report, for the first time, that TNF is present in TMJ fluid.

EXPERIMENTAL

Materials

Paramagnetic beads, 5 μm (M-450), coated with sheep anti-mouse IgG antibodies, and 2.85 μm (M-280), coated with either sheep anti-rabbit IgG antibodies or sheep anti-mouse IgG antibodies, the Dynabead rotator and the permanent magnet (Dynal MPC-E) were obtained from Dynal (Great Neck, NY, USA); human recombinant TNF-α (rTNF) and mouse anti-TNF MAbs (clone 199 and clone 195/8) were obtained from Boehringer-Mannheim (Indianapolis, IN, USA); rabbit serum, containing anti-TNF polyclonal antibodies (PAbs), was a gift from Dr. Anthony Cerami. The antibody fraction was purified using a Sepharose Protein A column (7 cm × 1 cm I.D.). The Sepharose Protein A, bovine serum albumin (BSA), human serum albumin (HSA), Thimerosol and phosphate-buffered saline (PBS) were obtained from Sigma (St. Louis, MO, USA). Interleukin-1 (Il-1) was a gift from Dr. Gloria Gronowicz. The Olympus-inverted microscope equipped with Hoffman phase optics and the Olympus camera were obtained from Micro-Tech (Hartford, CT, USA) and the Tech Pan 135 film was from Kodak (Rochester, NY, USA).

Attachment of antibodies to beads

Paramagnetic beads (5 μm) (500 μl containing 1.5-mg beads) coated with sheep anti-mouse IgG antibodies were mixed with clone 199 mouse anti-TNF MAb in an overnight incubation at 4°C with end-over-end rotation, in the Dynabead rotator, in 120 m*M* NaCl–2.7 m*M* KCl–10 m*M* phosphate, pH 7.4 (PBS) containing 0.1% BSA. The beads were recovered from solution with the

aid of the permanent magnet, then washed in four changes of PBS BSA with 30 min for each change. The bead–MAb complex was stored as a slurry in PBS–BSA with 0.01% Thimerosal. These MAb beads were washed and resuspended in PBS–BSA immediately before use. The concentration of the MAb beads in the slurry was determined by counting aliquots in a hemocytometer using 200 × total magnification.

Two types of 2.85-μm paramagnetic beads were used. The beads with sheep anti-rabbit antibodies were coated with purified rabbit anti-TNF PAbs while the sheep anti-mouse beads were coated with mouse anti-TNF MAbs, clone 195/8.

Calibration curve

A calibration curve was developed using rTNF as follows. $2 \cdot 10^5$ MAb-coated 5-μm beads were added to 60 μl of a PBS–BSA solution containing 1–4 ng of rTNF. After incubation for 2 min at room temperature, the beads were recovered, washed once with PBS–BSA and resuspended in 60 μl of PBS BSA. A second solution was prepared containing either PAb-coated or MAb-coated 2.85-μm beads at a concentration twenty-fold in excess of the number of large beads. Of this solution, 200 μl were added to the solution of 5-μm beads, the mixture was incubated at room temperature for 10 min, and the number of clusters was determined microscopically (see below).

Collection of TMJ fluid

Fluid was collected from eleven TMJs in eight patients undergoing either arthroscopy (seven TMJs) or arthrotomy (four TMJs) for internal derangements. All arthroscopy patients selected met the criteria stated in the position paper on arthroscopy of the TMJ by the American Association of Oral and Maxillofacial Surgeons. In addition, this study was approved by the Institutional Review Board of the University of Connecticut Health Center.

Fluid was collected prior to either arthroscopy or arthrotomy with the patient under general anesthesia. The superior joint space was first injected, by an inferolateral approach, using an 18-gauge needle with 1.5 ml of bupivacaine without epinephrine, followed by injection of 1.5 ml of lactated Ringers solution. After 1 min, all fluid was aspirated from the joint. The amount of fluid recovered ranged from approximately 250 to 1000 μl. The fluid was transferred to polypropylene tubes and stored at −20°C until assayed.

RESULTS

Separation of TNF from the TMJ fluid

A slurry of 5-μm paramagnetic beads coated with MAb specific for TNF (MAb beads) was prepared to contain $6.3 \cdot 10^6$ beads per ml in PBS–BSA. In most cases, 50 μl of the slurry were transferred to 100 μl of the TMJ fluid. In experiments designed to determine a suitable incubation time, the slurry remained in the fluid for as long as 30 min. After separation and quantification by the cluster assay (see below), we found that the amount of TNF retrieved after 2 min was similar to that recovered after 30 min. Therefore, in all subsequent experiments the slurry remained in the solution from 2 to 4 min. The beads were separated from the fluid by placing the test tube against a permanent magnet (MPC-E), and the solution was removed.

Definition of the cluster assay

After separation, the beads were resuspended in PBS- BSA, and a twenty-fold excess of either anti-TNF PAb- or MAb-coated 2.85-μm beads was added to each tube and incubated to allow a large bead–small bead complex, or cluster, to form. A diagrammatic representation of the configuration of this complex when PAbs were used is shown in Fig. 1A. Clusters were visualized and counted using an inverted microscope with Hoffman phase contrast optics at a total magnification of 400 ×. In Fig. 1B, a representative image obtained in the absence of TNF is shown. A single cluster, indicated by the arrow and defined as one large bead attached to one or more small beads, is visible. Fig. 1C shows an image obtained in the presence of TNF. Numerous clusters, indicated by the arrows, are visible.

For counting clusters, large beads were scored as being either isolated or in contact with a small-

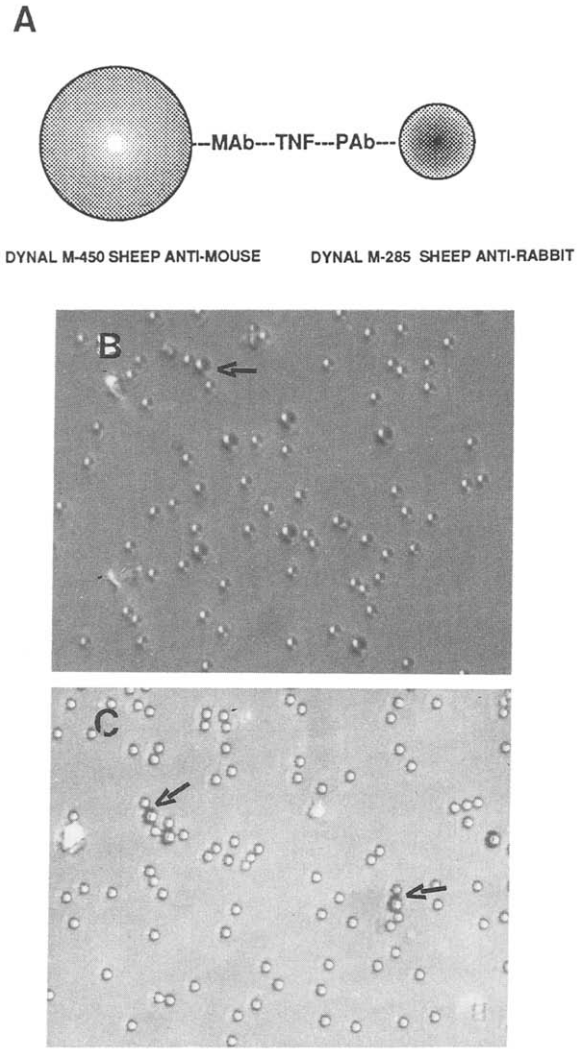

Fig. 1. Description of the cluster assay. (A) Schematic representation of the immunochemistry underlying the cluster assay. In the diagram, an MAb-coated bead is shown coupled to TNF which in turn is coupled to a PAb attached to a small bead. A cluster is formed when the PAb binds to the TNF–MAb bead complex. Photomicrographs show representative images of clusters and were obtained using an Olympus camera and Tech Pan 135 film. (B) A representative image obtained in the absence of TNF. A number of large beads and a greater number of small beads are visible. In this image, only one large bead was observed in contact with a small bead (arrow). (C) A representative image obtained in the presence of 4 ng of TNF. Several large beads (arrows) were observed in contact, or clustered with, small beads.

er bead. When counting, the initial microscopic field was chosen at random and after all large beads in that field had been scored, the microscope stage was moved to a contiguous field. The ratio of large beads found in clusters to the total number of large beads was then calculated. A sample processed without TNF served as the blank and, after the number of clusters was determined, a similar ratio was obtained. The blank ratio was subtracted from the experimental ratio to give the number of clusters which are expressed in arbitrary "cluster units" (CU).

Reproducibility of the procedure

The reproducibility of the procedure was studied as follows. To examine the reproducibility of counting, a stock solution (2 ng per 100 ml) of rTNF was prepared. Five aliquots were removed, processed as described above, and the number of clusters formed in each of the five samples was determined. Also, to examine the reproducibility of pipetting, five solutions, of five different concentrations (0, 0.5, 1, 2 and 4 ng) of rTNF were prepared, processed as described, and the number of clusters in each solution was determined. In both experiments, the arithmetic averages of the single counts of the five solutions or the single counts of the five different concentrations had a standard deviation (S.D.) of ± 2 CU.

Calibration curves

In order to evaluate the magnitude of the error associated with the cluster assay, the S.D. was determined as follows. In a representative experiment, standard solutions of rTNF were prepared at amounts indicated on Fig. 2. MAb beads (5 μm) were added, and the amount of TNF captured was determined by the cluster assay. Curves were generated by plotting the number of CU as function of TNF. For 1 ng, $n = 9$, for 2 ng, $n = 7$ and for 4 ng, $n = 8$; $r^2 = 0.998$ for the line. Fig. 2 shows both the means (squares) and the magnitude of the S.D. (error bars) for each of the eight values.

Specificity of the cluster assay

Body fluid samples would be expected to contain other compounds such as serum albumin and other closely related cytokines such as Il-1. Therefore, the specificity of the cluster assay pro-

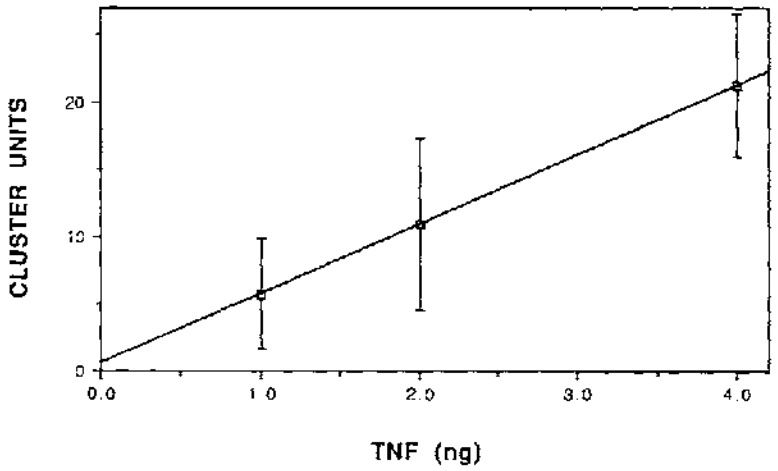

Fig. 2. Standard curve generated by scoring 200 large beads as positive or negative for cluster formation. In a representative experiment, standard solutions of TNF were prepared, at amounts indicated in a total volume of 60 μl. MAb beads were added and the amount of TNF captured was determined by the cluster assay. For 1 ng, n = 9; for 2 ng, n = 7 and for 4 ng, n = 8; r^2 = 0.998 for the line. Error bars represent the magnitude of S.D. for each value.

cedure was investigated by replacing TNF with either HSA or Il-1. In these experiments, solutions of various concentrations of HSA and IL-1 were prepared, and 5-μm MAb-coated beads were added followed by the addition of 2.85-μm PAb-coated beads. The clusters present at each of the concentrations were determined and the results are shown in Fig. 3. For comparison, the number of clusters recovered with various concentrations of added TNF is also shown on Fig. 3. In contrast to the number of clusters formed when TNF was present, Fig. 3 shows that no clusters were detectable with either HSA (open squares) or IL-1 (open circles).

Retrieval of TNF from TMJ fluid

After collection, 5-μm MAb-coated paramagnetic beads were introduced into the fluid, TNF captured on the bead surface and the beads separated from the fluid. Following resuspending and washing the beads, a twenty-fold excess of the smaller 2.85-μm antibody-coated beads was added, and the amount of TNF in each sample was measured by the cluster assay. Fig. 4 shows the distribution of TNF values obtained from each TMJ sample. The concentration ranged

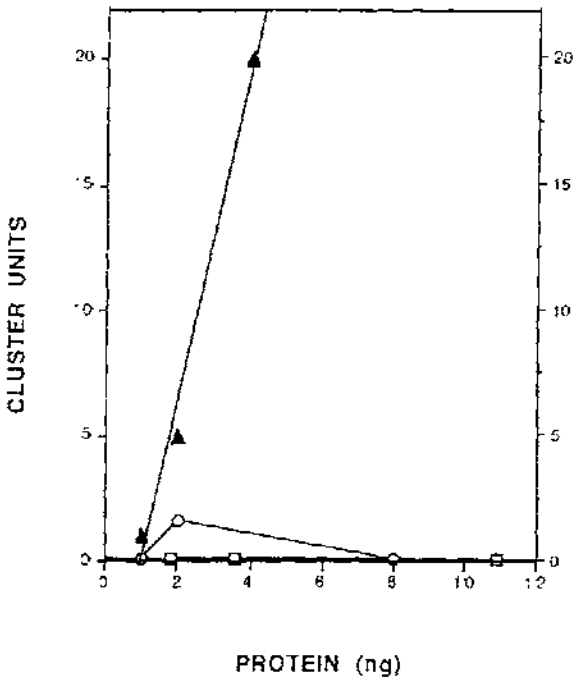

Fig. 3. Determination of Il-1 and HSA by the cluster assay. In this experiment, known amounts of Il-1 and HSA were used in place of TNF. Samples were incubated with 5-μm beads coated with anti-TNF MAbs. For the cluster assay, 2.85-μm beads were coated with purified anti-TNF PAbs. (□) HSA; (○) Il-1; (▲) TNF.

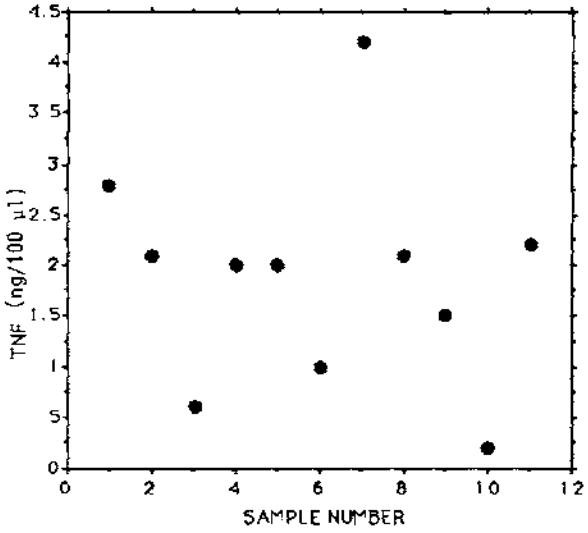

Fig. 4. Recovery of TNF from human TMJ synovial fluid. The results of the assay of eleven samples of human TMJ synovial fluid collected from eight patients undergoing either arthroscopy ($n = 7$) or arthrotomy ($n = 4$) are shown. An average value of 1.9 ng of TNF per 100 µl was obtained with an S.D. of ± 1.1 ng per 100 µl.

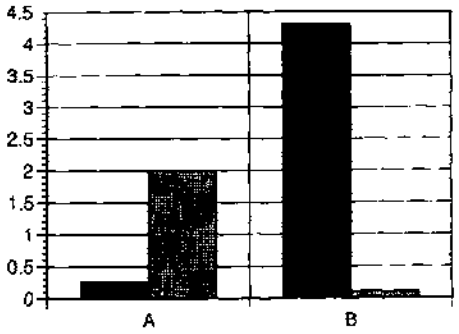

Fig. 5. Recovery of TNF. (A) A clinical sample was assayed and 0.2 ng of TNF was recovered. A second aliquot of this sample was "spiked" with 2 ng of authentic TNF and the sample assayed again. The recovery of TNF before (solid bar) and after (stippled bar) the spiking is shown. (B) Approximately 4 ng of TNF were recovered from a clinical sample using $2 \cdot 10^5$ beads. An additional $2 \cdot 10^5$ beads were added to the same sample and the TNF present was determined. The TNF values obtained following the first (solid bar) and second (stippled bar) collection are shown.

from a low of 0.2 ng to a high of 4.2 ng per 100 µl of fluid, with a mean value of 1.9 ng and an S.D. of 1.1 ng.

For those samples exhibiting low values of TNF, the possibility was considered that some component of the TMJ fluid was inhibiting the assay or otherwise inactivating the TNF. To test this possibility, a sample that was previously shown to have a low value, in this case 0.2 ng of TNF, was used. A 100-µl aliquot of this sample was then "spiked" with 2 ng of rTNF. Antibody-coated beads were added, and the amount of TNF recovered was determined by the cluster assay. As shown in Fig. 5A, over 95% of the TNF in the spiked solution was recovered in the assay, indicating that the original low cluster value was not due to the presence of an inhibitor.

Similarly, for those samples exhibiting high values of TNF, the possibility existed that additional TNF was present but not recovered because of an inadequate number of antibody sites on the 5-µm beads. To test this possibility, $2 \cdot 10^5$ 5-µm antibody-coated beads were added to a sample to determine the amount of TNF present. The beads were separated from the solution, and this solution was set aside. The beads were resuspended, 2.85-µm antibody-coated beads added, and the amount of TNF was determined. Next, to the solution that had been set aside an additional $2 \cdot 10^5$ 5-µm antibody-coated beads were added. Again the beads were separated from the solution, resuspended, and the amount of TNF recovered was determined. Fig. 5B presents the results of this experiment. As shown, about 4 ng of TNF were recovered by the first set of 5-µm antibody-coated beads. In contrast, the TNF recovered by the second set of antibody-coated beads was barely detectable. These findings indicate that almost all of the TNF that was originally present had been removed with the first set of beads and therefore a sufficient number of immunoadsorption sites were present initially on the 5-µm antibody-coated beads to assure recovery of greater than 95% of the TNF.

DISCUSSION

In the present study we used paramagnetic beads coated with anti-TNF MAbs as the solid phase to capture the TNF from the solution. Previous studies have used antibody-coated magnetic particles with open column chromatography to separate cell types [25]. Because of the microliter volumes of fluid that were available for the present study, it was advantageous to use a batch procedure. Batch separations have been used for the fractionation of creatine kinase isoenzymes using an anion exchanger [26], and anion exchangers were also used to separate lactate dehydrogenase isoenzymes using a batch technique [27]. A comparison of the efficacy of batch *versus* column chromatography has been presented [28].

The immunomagnetic procedures have other advantages as well. For example, the ability to separate the magnetic particles from the solution using a permanent magnetic field provided a convenient method for concentrating the TNF. The ability to concentrate the sample ensures that sufficient amount of antigen will be available for the analysis. And, finally, another advantage is that the use of MAbs insures that the separation will be specific. This specificity was demonstrated by the results of experiments which showed that neither the related cytokine Il-1 nor HSA would be captured.

Following immunomagnetic separation, the cluster assay was used to quantify the amount of TNF. The cluster assay, while similar to an ELISA, replaces the color-generating system with a second smaller bead. In the cluster assay, the smaller bead can be coated with either PAbs or MAbs, provided the latter are reactive to epitopes different from the MAbs attached to the 5-μm capture beads. With the cluster assay, a positive result is obtained when the antibodies on the small beads bind to an epitope on the TNF forming a bead MAb–TNF–antibody–bead cluster. As the two beads are different in size, the contact point between a large and small bead can be taken as evidence of the presence of a single TNF molecule and a one-to-one correspondence may be set up between the number of contact points and the number of TNF molecules.

When compared to assays such as the ELISA and the latex agglutination assay, the cluster assay has several advantages. For example, the antigen need not be removed from the bead for analysis. Also, the ELISA procedure requires the addition of multiple reagents, and as each addition increases the possibility of error, the degree of precision of the method will be reduced. In contrast, the cluster assay involves the addition of only one reagent, the solution of small beads. Thus, the cluster assay will be more precise than the ELISA.

The latex agglutination assays, while superficially similar to the cluster assay, have several significant differences. For example, agglutination assays involve polymerization of smaller units to form a structure of sufficient size to be detected by a method such as nephelometry. In contrast, with the cluster assay only a dimer is formed and each dimer indicates the presence of an antigen.

While the immunomagnetic separation method has the advantages described above, there are some limitations. The most significant is that the fluid must be collected from the body space before separation. As this collection involves invasive surgical procedures, the possibility must be considered that the collection procedure might influence the amount of antigen recovered. For example, the amount of TNF recovered might be affected by the introduction of surgical instruments and the washing procedure necessary to collect the fluid. Overcoming this limitation would require the development of procedures to collect the antigen directly in the body cavity without the collection of fluid. Procedures for the *in situ* separation of TNF are described in the accompanying paper [29].

ACKNOWLEDGEMENTS

We wish to thank Drs. Keith Rogerson and Leon Assael for providing clinical specimens. This research was supported by grants from Molecular Technologies, Inc. and the University of Connecticut Research Foundation.

REFERENCES

1 E. F. Rossomando, *Methods Enzymol.*, 182 (1990) 309.
2 J. Vilcek and T. H. Lee, *J. Biol. Chem.*, 266 (1991) 7313.
3 E. F. Rossomando, J. Kennedy and J. Hadjimichael, *Arch. Oral Biol.*, 35 (1990) 431.
4 R. A. Kozol, E. F. Rossomando, M. Prasad and R. Jaszewski, *Surg. Res. Commun.*, 9 (1990) 311.
5 K. E. Safavi and E. F. Rossomando, *J. Endodontol.*, 17 (1990) 12.
6 H. R. Michie, K.R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff and D. W. Wilmore, *N. Engl. J. Med.*, 318 (1988) 1481.
7 F. S. DiGiovine, G. Nuki and G. W. Duff, *Ann. Rheum. Dis.*, 47 (1988) 768.
8 E. Girardin, G. E. Grau, J. M. Dayer, P. Roux-Lombard, J5 Study Group and P.-H. Lambert, *N. Engl. J. Med.*, 319 (1988) 397.
9 J. A. DeBoever, in G. A. Zarb and G. E. Carlsson (Editors), *Temporomandibular Joint Function and Dysfunction*, Mosby, St. Louis, MO, 1979, p. 193.
10 S. Kopp, B. Wennerberg and E. Clemensson, *Scand. J. Dent. Res.*, 91 (1983) 33.
11 J. H. Quinn and N. G. Bazen, *J. Oral Maxillofacial Surg.*, 48 (1990) 968.
12 R. I. Brooke, P. G. Stenn and K. J. Mothersill, *Oral Surg.*, 44 (1977) 844.
13 C. B. Laskin, *J. Am. Dent. Assoc.*, 79 (1969) 147.
14 L. Vestergaard-Christensen, *J. Oral Rehabil.*, 5 (1978) 23.
15 W. G. Shipman, C. S. Greene and D. M. Laskin, *J. Psychosom. Res.*, 18 (1974) 475.
16 J. M. Dayer, B. Beutler and A. Cerami, *J. Exp. Med.*, 162 (1985) 2163.
17 E. F. Rossomando, G. Groniwicz, J. Hadjimichael and R. B. Rutherford, *J. Leukocyte Biol.*, 42 (1987) 558.
18 D. R. Bertolini, G. E. Nedwin, T. S. Bringmani, D. D. Smith and G. R. Mundy, *Nature*, 319 (1986) 516.
19 G. P. Vonk and J. L. Schram, *J. Immunol. Methods*, 137 (1991) 133.
20 P. Kronick and R. W. Gilpin, *J. Biochem. Biophys. Methods*, 12 (1986) 73.
21 T. Lea, F. Vartdal, C. Davies and J. Ugelstad, *Scand. J. Immunol.*, 22 (1985) 207.
22 F. Vartdal, G. Kvalheim, T. E. Lea, V. Bosnes, G. Gaudernack, J. Ugelstad and D. Albrechtsen, *Transplantation*, 43 (1987) 366.
23 C. P. Reynolds, A. T. Black, J. W. Saur, R. C. Seeger, J. Ugelstad and J. N. Woody, *Transplant Proc.*, 17 (1985) 434.
24 C. S. Owen, in T. G. Pretlow, II and T. P. Pretlow (Editors), *Cell Separation*, Vol. 2, Academic Press, Orlando, FL, 1983, p. 127.
25 C. S. Owen and P. A. Liberti, in T. G. Pretlow, II and T. P. Pretlow (Editors), *Cell Separation*, Vol. 4, Academic Press, Orlando, FL, 1983, p. 259.
26 L. G. Morin, *Clin. Chem.*, 22 (1976) 92.
27 M. P. Menon, S. Miller and B. S. Taylor, *J. Chromatogr.*, 378 (1986) 450.
28 D. Reichenberg, in C. Calmon and T. R. E. Kressman (Editors), *Ion Exchangers in Organic and Biochemistry*, Interscience, New York, 1957, p. 86.
29 E. F. Rossomando, L. B. White and J. Hadjimichael, *J. Chromatogr.*, 583 (1992) 19.