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Immunomagnetic separation of tumor necrosis factor α

II. *In situ* procedure for the human gingival space

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ABSTRACT

An *in situ* procedure has been developed for the separation of tumor necrosis factor (TNF) α directly from the fluid in the human gingival space. Paramagnetic beads coated with anti-TNF monoclonal antibodies were introduced into the gingival space of the subject with a polypropylene-tipped calibrated delivery system and retrieved using a permanent magnet designed to fit into the space. After retrieval, the amount of immunoadsorbed TNF was quantified using an immunochemical assay called the "cluster assay". The results indicate that following the appropriate preparation of the site, over 95% of the beads could be recovered. With this method we found that 62% of those cavities sampled contained TNF and that the values ranged from 0.10 to 13.0 ng/ml with a mean value of 1.7 ng/ml. A comparison of these values with those obtained from the same space using other methods suggests that the immunomagnetic method was more effective in retrieval of TNF. Because the separation is performed *in situ* we have named the procedure "chromatobiosis".

INTRODUCTION

Living organisms contain many body cavities or spaces that contain fluid. To date, chromatographic studies on these body fluids require the removal of the fluid from the space and its analysis outside the subject. In the present study we have developed a procedure for separation *in situ*. Rather than collect the fluid and then perform the chromatography, a solid support is introduced directly into the space. In the present study we have used *in situ* separation to isolate tumor necrosis factor (TNF) α from the gingival crevice or space. This space surrounds each tooth and when healthy, is approximately 3 mm long × 2 mm deep × 0.1 mm wide, and if this space is completely filled, it would contain about 60 μl of fluid. This space may be entered for purposes of collecting the fluid within and previous studies have used paper strips or capillary tubes for this purpose. For a review of the anatomy and methods used previously, see ref. 1. In previous studies, using different methods, we recovered TNF, a 17 000 relative molecular mass protein, from this space [2,3]. The biochemistry and biological effects of TNF have recently been reviewed [4].

As reported in ref. 5 we used, as the solid phase, paramagnetic beads precoated with anti-TNF monoclonal antibodies (MAbs). We have introduced a slurry of these immunobeads into the space and retrieved them with a magnetic harvester, designed to fit into the space. After recovery, the amount of bead-bound TNF was

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quantified using the cluster assay also described in ref. 5.

The results of the present study indicate that it is possible to introduce and retrieve immunobeads from a body cavity. Because this represents a new type of separation we have given this procedure the name "chromatobiosis" to refer to the process of *in situ* separation.

EXPERIMENTAL

Materials

Paramagnetic beads, 5 μm (M-450) and 2.85 μm (M-280), coated with sheep anti-mouse antibodies were obtained from Dynal (Great Neck, NY, USA); human recombinant TNF-α (rTNF) and mouse anti-TNF MAbs (clone 199) were 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), Thimerosal and phosphate-buffered saline (PBS) were obtained from Sigma (St. Louis, MO, USA). The Olympus-inverted microscope equipped with Hoffman phase optics and the Olympus camera were obtained from MicroTech (Hartford, CT, USA) and the Tech Pan 135 film was from Kodak (Rochester, NY, USA). The 0.5–10 μl digital Finn pipette was from Flow Labs. (McLean, VA, USA) and the microcapillary and sequencing gel-loading tip with 0.3-mm flat tip, T4000, was from Marsh Biomedical (Rochester, NY, USA). The disk-shaped neodymium–iron–boron magnets, B35-104 (1800 gauss per magnet), were obtained from Edmund Scientific (Barrington, NJ, USA).

Attachment of antibodies to beads

Mouse anti-TNF MAb was attached to the 5-μm paramagnetic beads (500 μl containing 1.5-mg beads) precoated with sheep anti-mouse IgG antibodies as described [5]. The concentration of the MAb-coated beads was determined by counting an aliquot in a hemocytometer using 200 × magnification.

Polyclonal anti-TNF beads were prepared using procedures described [5] by attaching purified rabbit anti-TNF PAbs to 2.85-μm paramagnetic beads precoated with sheep anti-rabbit IgG MAbs.

Design and fabrication of the harvester device

An instrument for generating a magnetic field was fabricated from steel. It had a pencil style shank (7.5 cm × 8 mm) and a removable sterilizable spatula tip (2 cm × 2 mm) held to the shank by a set screw. Four disk-shaped neodymium-iron–boron magnets were placed in the shank about 2 cm from the spatula end of the shank. The magnetic field generated at the tip was between 185 and 188 gauss.

Quantitation of TNF on the beads by cluster assay

After retrieval, the beads were vortex-mixed from the harvester tip into a 1.5-ml Eppendorf microcentrifuge tube, insoluble debris was disrupted by gentle homogenization with microcentrifuge pestles and removed by washing three times with PBS–BSA, and the beads were processed for the cluster assay as previously described [5]. In brief, a twenty-fold excess of PAb-coated beads were added to each tube and incubated to allow a bead MAb TNF PAb small bead complex, or cluster, to form. Clusters were visualized and counted as described [5]. Cluster units were converted to TNF amounts using a calibration curve. The specificity of the cluster assay procedure has been reported [5]. Neither human serum albumin nor another cytokine, namely interleukin-1 (Il-1) formed clusters [5].

Study population

Samples were collected from twenty-one spaces in eight out-patients presenting for treatment at the University of Connecticut School of Dental Medicine. Individuals were between the ages of 30 and 70 years. Clinical signs and/or symptoms of systemic disease resulted in exclusion. In addition, this study was approved by the Institutional Review Board of the University of Connecticut Health Center. Prior to sample collection, supragingival plaque was removed, the tooth surface gently washed with water, dried

with air and isolated with cotton rolls to minimize contamination with saliva.

RESULTS

Introduction of beads into the gingival crevice

A slurry of 5-μm paramagnetic beads with MAbs for TNF (MAb beads) was prepared to contain $1.5 \cdot 10^6$ beads per ml. In most cases, 4–5 μl of the slurry were introduced into the gingival space using the Finn pipette. As shown in Fig. 1a, the flat tip of the pipette was placed into the space. The arrow on Fig. 1a indicates the slurry in the pipette tip. The slurry was dispensed slowly into the space (Fig. 1b). In experiments designed to determine a suitable residence interval, the

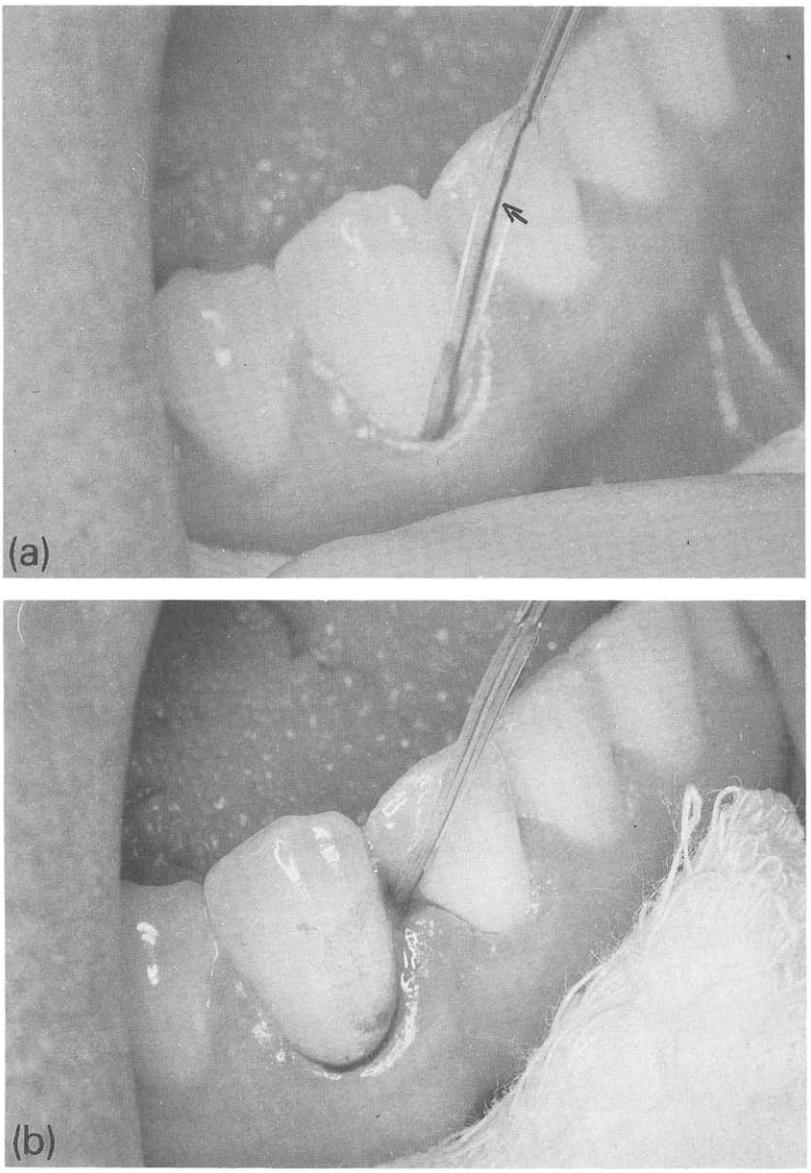

Fig. 1. Introduction of immunobeads in gingival space. (a) Introduction of the pipette tip into the gingival space. A slurry of immunobeads was prepared to contain $1.5 \cdot 10^6$ beads per ml, and the pipette was filled with 4 μl. The end of the pipette tip has been placed about 1 mm into the space. The average depth of this space was about 2 mm. The arrow points to the brown-colored slurry of the immunobeads inside the pipette tip. (b) Dispersal of the immunobeads into the space. The slurry was dispensed into the space slowly and carefully to minimize overflow.

slurry remained in the crevice for 0.5, 1, 2 or 3 min. After retrieval and quantitation by the cluster assay, we found that the amount of TNF retrieved after 0.5 min was similar to that recovered after 3 min. Therefore, in all subsequent experiments the slurry remained in the space from 0.5 to 1 min.

Retrieval of beads from the gingival crevice

The beads were retrieved by placing the tip of the magnetic harvester into the space (Fig. 2a). The magnetic properties of the beads and harvester tip allow the beads to be collected from the space with only a gentle sweeping motion. Fig. 2b shows the harvester after retrieval with the beads.

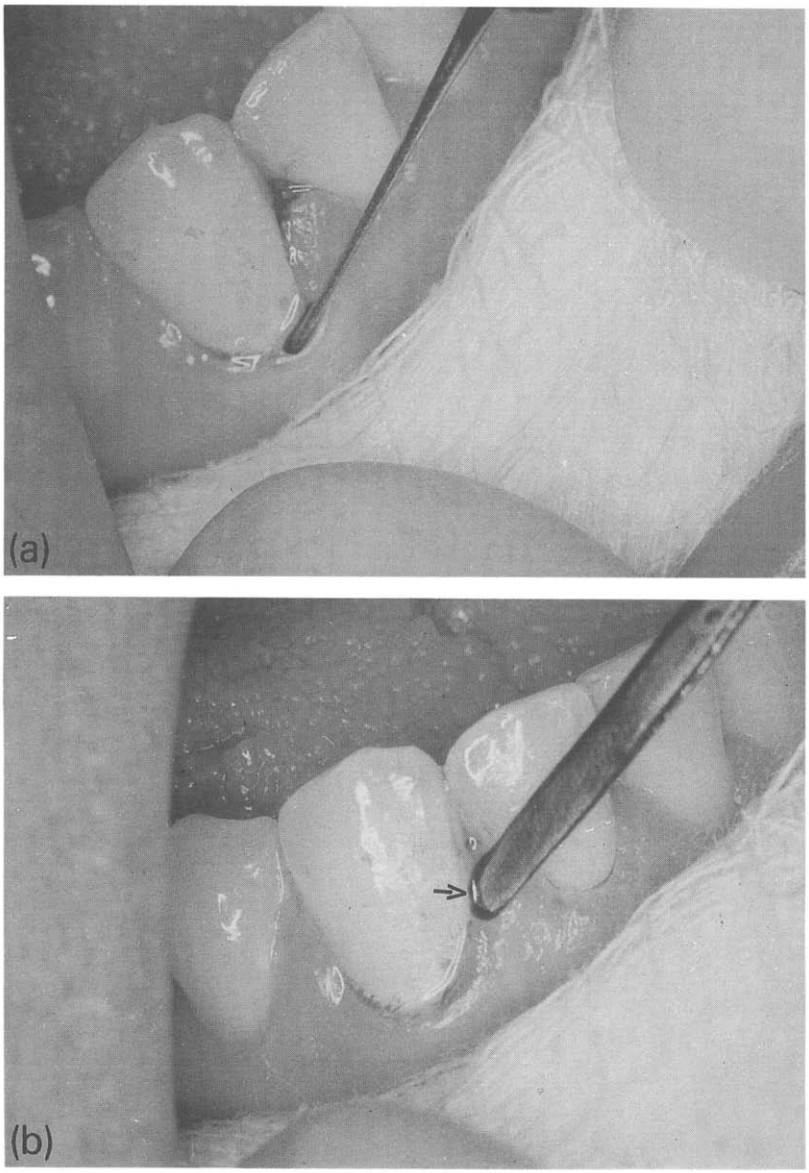

Fig. 2. Retrieval of immunobeads from gingival space. (a) Placement of magnetic harvester into the space. The flat tip of the magnetic harvester has been placed about 1 mm into the space. (b) Retrieval of the immunobeads with the magnetic harvester. The beads were retrieved from the space by the tip of the magnetic harvester with a gentle sweeping motion. At the end of the retrieval process, the beads adhere to the end of the harvester tip as a dark mass indicated by the arrow.

the dark mass indicated by the arrow, adhering to the tip. The beads were removed from the harvester by placing the harvester tip into an Eppendorf tube (1.5 ml) containing 100 μl of PBS buffer, and gently vortex-mixing the solution. The beads were retrieved from the solution with the MPC-E magnetic holder, washed and processed for the cluster assay as described [5].

Efficiency of bead retrieval

Experiments were undertaken to determine those parameters that affected recovery of beads from the space. For these experiments the same subject was used, and once or twice per week a fixed number of beads was introduced into the same site. Beads were recovered with the harvester and counted using a hemocytometer. The number recovered was compared to the number introduced and the fraction expressed as a percentage. Experiments were performed each week, for a total of five samples over a three-week period. All retrieval experiments were performed by the same clinician.

While in most instances, greater than 95% of the beads were recovered, the inability to recover all of the beads could be accounted for by some beads adhering to a mucoid material at the site. Removal of this material prior to the slurry being dispensed into the space resulted in recovery of greater than 95% of the beads (data not shown).

Quantitation of TNF from human subjects

The immunobeads that were introduced into the space were retrieved, and the immunoadsorbed TNF on the beads quantitated by the cluster assay. Two representative images of samples are shown in Fig. 3a and b. The sample marked in Fig. 3a shows clusters, indicated by the arrow, as well as single large beads and single small beads while the sample marked in Fig. 3b shows no clusters. Some debris is visible. Paramagnetic beads were introduced into twenty-one spaces. As shown in Table I, TNF was recovered from 62% of these spaces. Table I also shows that with paper strips and enzyme-linked immunosorbent assay (ELISA) for quantitation only 21% of the spaces were positive. Finally, Table I shows that when immunobeads were combined with an ELISA, 65% of the spaces were positive.

When TNF was recovered, as shown in Table II, the values ranged from 0.1 to 13.0 ng/ml of gingival fluid with an average value of 1.7 ng/ml. In contrast, with paper strips TNF values ranged from 0.003 to 17 ng/ml with an average of 0.06

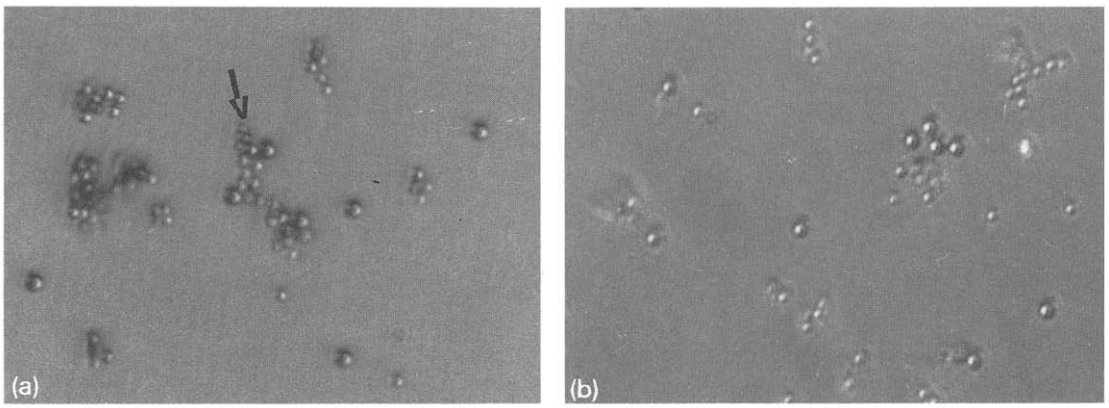

Fig. 3. Immunobeads after recovery from gingival space and during cluster assay. A slurry containing $1.5 \cdot 10^8$ immunobeads per ml was prepared, 4 μl were introduced into the gingival space as illustrated in Fig. 1, and retrieved using the magnetic harvester as illustrated in Fig. 2. The immunobeads were removed from the harvester tip and processed for the cluster assay as previously described [5]. (a) and (b) are microscopic images obtained from two different samples. Single large beads and single small beads are seen. The arrow indicates the presence of a cluster. Some debris is also visible.

TABLE I

COMPARISON OF RESULTS: TNF RECOVERED FROM GINGIVAL SPACES

Study	Total sites	Sites positive[a] (%)	Ref.
Paper strips/ELISA	162	21	2
Bead capture/bead-based ELISA	26	65	3
Bead capture/bead-based cluster assay	21	62	–

[a] All values have been rounded off to the nearest whole number.

ng/ml. And finally, when the immunobeads were used together with an ELISA, the TNF values ranged from 0.1 to 2.0 ng/ml with an average of 0.65 ng/ml.

DISCUSSION

In the present study, TNF was separated *in situ* from the fluid in the gingival space using immunomagnetic beads coated with anti-TNF MAbs introduced directly into the space. The amount of bead-bound TNF was determined using the cluster assay described in ref. 5. The results obtained by this method have been compared to those obtained with another *in situ* method for capture and retrieval, namely paper strips, and another method for quantitation, an ELISA. Although the sample size is too small to perform meaningful statistical analysis, an attempt has been made to evaluate both the precision and accuracy of these data.

TABLE II

COMPARISON OF TNF VALUES IN THE GINGIVAL SULCUS BY SEVERAL METHODS

Collection method	Assay method	TNF[a] (ng/ml)	Ref.
Paper strips	ELISA	0.003[b] 1? (0.06)	2
Immuno adsorption	ELISA	0.10–2.0 (0.65)	3
Immuno adsorption	Cluster	0.10 13.0 (1.7)	–

[a] Average values are shown in parenthesis. In calculating the concentration of TNF the volume of fluid present in the space at the time of assay has been calculated to be 0.1 ml from the dimensions of a representative space [1].

[b] This value was estimated by extrapolation of a standard curve. Therefore its accuracy is questionable.

Ideally, as part of an evaluation of the accuracy, recovery studies would be carried out where known amounts of TNF would be added to the space, retrieved and assayed. However, as the effects of TNF added to the space are unknown and therefore might be detrimental, it was not possible to obtain permission to perform such experiments on human subjects. Alternative experiments, in which known amounts of TNF were resuspended in previously removed sulcular fluid, were also not considered representative of the procedure since in the present method sulcular fluid is not actually removed. And, finally, it should be noted that as the studies from this laboratory are the first to report on the recovery of TNF from this space, there are at the present time no "correct" or true values. In fact, based on the random method in which subjects were chosen, the condition of the space and therefore the amount of TNF recovered would be expected to span a range of values as would be the case with other biologicals in body fluids. Therefore, an evaluation of the accuracy of the values obtained in these studies must await the outcome of more extensive and controlled clinical studies.

However, as an alternative to evaluate the accuracy of the method, the number of sites determined to be positive by the immunobead method may be compared to the number determined by

another method. Using paper strips to access the gingival space, we reported that TNF was recovered from only 21% of the sites [2]. In contrast, when immunomagnetic beads were used we found the number of sites positive to be 62% in the present study, and 65% in a previous study [3]. As TNF has been associated with inflammation, and as at least 50% of sites chosen at random would be expected to show some signs of inflammation, the larger number of positive sites obtained with the use of the MAb-coated beads suggests that this method facilitates recovery and would, in principle, be better able to provide more accurate data.

The precision of the values obtained in any measurement is a function of the number of steps involved in the measurement and the precision of each of the several steps. Clearly, the greater the number of steps to complete the measurement, the greater the opportunity for error and the lower the precision. The present methods have two steps where errors could affect the precision of the measurement: retrieval of the TNF and the analytical procedure by which the amount of TNF is measured.

The difficulties associated with retrieval of TNF by paper strips include non-specific capture and the necessity to remove the TNF from the strip for assay [2]. In contrast, with the immunobead-based method, the capture is specific and the TNF can be assayed direclty on the bead. Also, the ELISA requires more steps than the cluster assay to complete the analysis. For example, with the ELISA the antigen must be first attached to the plate-bound antibody; with the cluster assay the TNF is already bound to the bead. Also, the ELISA requires a series of reagents in order to produce a "color" which, in turn, requires conversion to optical density. In contrast, with the cluster assay only one reagent is added, namely, the Pab-coated smaller beads, and the number of clusters can be determined directly from the optical image. Given the fewer number of steps for both retrieval and analysis, in theory, the immunobeads method should be more precise.

The values summarized in Table II tend to support this conclusion by showing that with immunobeads higher average values were reported than with paper strips. In addition, the *in situ* values reported in the present study compare favorably with the *in vitro* studies reported previously. For example, TNF in serum ranged from 0.007 to 0.040 ng/ml [6] and, in another study, it was 0.15 ng/ml [7]. As this serum was obtained from "healthy" patients, these values represent some "normal" values. In contrast, TNF from rheumatoid synovial joint fluid ranged from 0.25 to 2.6 ng/ml [8]. Also, TNF from periapical abscesses ranged from 24 to 4900 ng/ml [9], while TNF in gastric fluid had an average value of 0.34 ng/ml [10] and, finally, as reported in ref. 5, temporomandibular joint fluid had values ranging from 2 to 42 ng/ml.

In this paper we report that immunobeads may be introduced and retrieved from a body cavity. While immunomagnetic separation has been used previously, reports have been limited to the *in vitro* use of this technology [11–14]. To our knowledge, this report is the first to describe its use *in situ*. As this study may also be the first report of "chromatography" performed directly on a subject, we have named this process of *in situ* separation "chromatobiosis" from the two roots "chromato" for separation and "biosis" for living. This study suggests that by modification of the instrumentation and appropriate choice of antibodies, chromatobiosis might be applicable to the recovery of a variety of compounds from other body cavities.

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