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Intraperitoneal immunization of human subjects with tetanus toxoid induces specific antibody-secreting cells in the peritoneal cavity and in the circulation, but fails to elicit a secretory IgA response

C. LUE*†, A. W. L. VAN DEN WALL BAKE*, S. J. PRINCE*, B. A. JULIAN†, M.-L. TSENG*, J. RADL‡, C. O. ELSON*† & J. MESTECKY*† *Departments of *Microbiology and †Medicine, University of Alabama at Birmingham, Birmingham, AL, USA, and ‡IVVO-TNO, Leiden, The Netherlands*

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SUMMARY

Five patients on continuous ambulatory peritoneal dialysis (CAPD) were immunized intraperitoneally with tetanus toxoid (TT) through an indwelling catheter. Four control patients on CAPD received the same dose of TT intramuscularly. Before immunization, virtually no anti-TT antibody-secreting cells (AbSC) were detected by the enzyme-linked immunospot (ELISPOT) assay in peripheral blood or peritoneal fluid from patients of either group. One to 2 weeks after immunization, high frequencies of TT-specific AbSC were detected in the circulation and peritoneal cavity. More than 80% of those cells were of the IgG isotype, with IgA accounting for most of the remainder. Patients receiving TT by the i.p. route showed significantly higher frequencies of specific IgG and IgA AbSC in the peritoneal cavity than patients immunized intramuscularly. Frequencies of AbSC in peripheral blood did not significantly differ between the two groups. Immunization with TT by both routes resulted in a significant increase of IgG anti-TT antibodies in serum, saliva and peritoneal fluid. A significant IgA antibody response was seen only in serum and peritoneal effluents. Therefore, i.p. immunization of human subjects with TT elicited both a localized response in the peritoneal cavity as well as a systemic response in serum, but did not induce a salivary IgA response.

Keywords IgA peritoneal immunization secretory immunity tetanus toxoid antibody-secreting cells

INTRODUCTION

Anatomical studies suggested a role of the mammalian peritoneum in immunological processes. Attention has primarily focused on the greater omentum, where 'milky spots' were first described by von Recklinghausen in rabbits [1]. Milky spots in human greater omentum were first described by Seifert in 1921 [2]. Comparative studies on milky spots in various experimental animals showed that they consist of an accumulation of macrophages and lymphocytes with or without blood vessels [3]. Trebichavsky *et al.* [4] showed that the development of the lymphatic apparatus of the pig omentum is closely related to the general immunological status of the animal, and not solely due to i.p. irritation. Recently, Shimotsuma and colleagues [5] have characterized the cellular composition of human omental milky spots with modern histochemical techniques. Macrophages were the most numerous cellular component, followed by B cells, T cells and mast cells. The number of milky spots is highest in infancy with 20–40/cm^2 and gradually decreases to 5–14/cm^2 at 3–10 years of age, and less than 5/cm^2 at 10–78 years of age [6]. Mesothelial stomata overlying omental milky spots allow milky spot lymphocytes to enter and possibly also to leave the peritoneal cavity [7]. These data suggest that milky spot cells play a role in the immunological defence of the peritoneal cavity.

Intraperitoneal injection of antigen in animals has been frequently used not only for parenteral immunization, but also for induction of secretory IgA antibody responses [8–10]. The reason for an effective induction of secretory IgA responses was explained by Kroese *et al.* [11], who showed that B cells from the murine peritoneal cavity repopulated the intestinal lamina propria of recipient mice with IgA-secreting cells. Based on these experiments, it was estimated that up to 50% of murine intestinal IgA-secreting cells were derived from surface IgA-negative precursors in the peritoneal cavity. Many of those precursors were self-replenishing B1 (CD5$^+$) cells which comprise a majority of B cells in the murine peritoneal cavity [11,12]. Recently, Solvason & Kearney [13] have demonstrated that analogous cells are also found in the human fetal omentum, which may serve as an additional site of B cell generation. Casali

Correspondence: Dr Cummins Lue, University of Alabama at Birmingham, Department of Microbiology, UAB Station, Birmingham, AL 35294, USA.

Table 1. Clinical and demographic features of human subjects with end-stage renal disease (ESRD) enrolled in the study

Patient no.	Age	Sex	Race	Cause of ESRD	Duration of ESRD (years)	Duration on CAPD (years)	Route of immunization
1	19	Male	Black	Chronic glomerulonephritis	1·5	1	i.p.
2	51	Female	Black	Hypertension	2·5	2	i.p.
3	57	Female	Black	Hypertension	3	3	i.p.
4	52	Female	Black	Hypertension	1	1	i.m.
5	25	Female	White	Unknown	2	1·5	i.m.
6	21	Male	Black	Focal sclerosing glomerulopathy	2	2	i.p.
7	56	Female	Black	Hypertension	2	2	i.m.
8	61	Female	Black	Diabetes mellitus	3·5	3·5	i.p.
9	41	Female	Black	Membranoproliferative glomerulonephritis	2	1	i.m.

Median age, 51 years. Median duration of ESRD, 2 years. Median duration on CAPD, 2 years.
CAPD, Continuous ambulatory peritoneal dialysis.

& Notkins have examined the antibody repertoire of human CD5$^+$ B cells and found that it includes a variety of self-antigens, e.g. double-stranded DNA, and foreign antigens, such as tetanus toxoid (TT) and bacterial lipopolysaccharide (LPS) [14].

We investigated whether peritoneal precursors of mucosal IgA plasma cells exist also in the human peritoneal cavity. Studies of the human peritoneal cavity are limited because of medical and ethical constraints. Patients on continuous ambulatory peritoneal dialysis (CAPD) represent the only group of human subjects with a prolonged access to the peritoneal cavity through an indwelling catheter [15]. Several investigators have studied the cellular components of the peritoneal effluents [16–19]. Macrophages and lymphocytes account for the majority of cells, followed by smaller percentages of neutrophils, eosinophils and basophils. The lymphocyte population consists of T, B, and natural killer (NK) cells. Both helper and suppressor T cell phenotypes were identified. One investigator determined the presence of surface immunoglobulin on peritoneal B cells and found an isotype distribution of 17% IgG, 25% IgA, and 10% IgM [20]. Interestingly, the numbers of IgA-bearing B cells declined after two or more episodes of peritonitis. However, B cells were not examined for the expression of CD5.

We examined B cells obtained from peritoneal effluents for their surface immunoglobulin isotype and the ability to secrete immunoglobulin. We present results of experiments in which patients were intraperitoneally immunized with TT, a purified protein antigen. TT is a safe and well defined antigen against which human B cells produce specific antibody, either constitutively or after vaccination with TT [14]. Although anti-TT antibody responses have been shown to be predominantly of the IgG class, our group and others have found a significant polymeric IgA response to parenteral immunization of normal human subjects to TT [21–23]. The immune responses in the peritoneal cavity and in the systemic circulation were studied. The question whether i.p. immunization leads to the induction of specific antibodies in external secretions was addressed.

SUBJECTS AND METHODS

Human subjects

This study was approved by the Institutional Review Board at the University of Alabama. Nine patients, seven females and two males, with end-stage renal disease on CAPD were recruited and provided informed consent. Table 1 shows some clinical features of the subjects. Table 2 shows entry criteria. Premature exit criteria were development of peritonitis or allergic reaction to the immunization.

Table 2. Entry criteria for human subjects

1. Age 18 years or older
2. End-stage renal failure and on continuous ambulatory peritoneal dialysis (CAPD)
3. No episodes of peritonitis within the last 2 months
4. No current infection of the exit site of the peritoneal dialysis catheter
5. History of primary immunization with tetanus toxoid
6. No tetanus immunization (either primary or booster) within the last 5 years
7. Negative serology for hepatitis B virus infection
8. No clinical evidence for infection with HIV
9. No exposure to topical or systemic immunosuppressive therapy (glucocorticosteroids or cytotoxic drugs) within the last 12 months
10. Haematocrit greater than 22%
11. No history of allergy to tetanus toxoid, antibiotics, other drugs, or foodstuffs

Immunization

The patients were randomized into two groups. Five subjects were administered one 0·5-ml dose of TT (Tetanus Toxoid Ultrafined, Wyeth-Ayerst, Marietta, PA) each through the CAPD catheter after draining the overnight dialysis fluid. Isotonic saline (250 ml) was instilled through the catheter to ensure deposition of the vaccine into the peritoneal cavity. The regular CAPD schedule was resumed after 8 h. Four subjects served as controls and received one 0·5-ml dose of the TT vaccine intramuscularly in the deltoid muscle. Blood, saliva, and peritoneal effluents (the overnight dialysate) were obtained before, 7, 14, and 21 days after immunization.

Cells

Venous blood was collected in sterile, heparinized syringes. Peripheral blood lymphocytes (PBL) were isolated by centrifu-

gation on a Ficoll–Hypaque (Sigma Chemical Co., St Louis, MO) density gradient. The cells were washed three times in Dulbecco's PBS (GIBCO, Santa Clara, CA) and resuspended in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin.

The entire overnight peritoneal dialysate as the source for peritoneal lymphocytes was collected by gravity drainage. Peritoneal lymphocytes were obtained by centrifugation of the fresh effluent at 300 *g* for 15 min [16,18]. After discarding the supernatant, the cell pellet was washed twice with Dulbecco's PBS. Cells were resuspended in complete medium. Viability was checked by trypan blue exclusion. A differential cell count was performed after Wright stain, and all cell preparations were analysed freshly for immunofluorescence staining, flow cytometry, or ELISPOT assay.

Body fluids

Serum was obtained by centrifugation of clotted whole blood. Peritoneal effluents were used for measurements of antibody levels after removal of cellular elements by centrifugation as outlined above. Whole saliva was collected by asking patients to drool into a cup [24]. All body fluids were stored frozen at −20°C until assayed.

ELISPOT

Peripheral blood and peritoneal cells were assayed for total immunoglobulin or antibody-secreting cells (AbSC) in an ELISPOT assay, as described previously [25–27]. Briefly, purified TT (kindly provided by Wyeth Laboratories, Marietta, PA) was used as the coating reagent in the antigen-specific ELISPOT. For the detection of TT-specific AbSC, individual wells of the Millititer HA plate (Millipore, Bedford, MA) were coated with 100 μl of TT at 10 μg/ml overnight at 4°C. On the following day, remaining binding sites were saturated with 200 μl culture medium containing 10% FCS for 30 min at 37°C in a humidified atmosphere with 5% CO_2. After discarding the blocking solution, 100 μl of cell suspension were dispensed into the wells at different cell densities in duplicate. After incubating the cells for 3–4 h at 37°C and 5% CO_2, the plate was rinsed three times with PBS and three times with PBS containing 0·05% Tween 20 (Sigma). Biotinylated goat anti-human IgA, IgG or IgM (Tago, Burlingame, CA) diluted 1:750 in PBS/Tween were added as secondary antibody. After an overnight incubation and thorough washing, avidin-alkaline phosphatase (Sigma) was added to the wells at 1·0 μg/ml to bind the secondary antibody at room temperature for 60 min. Following a final wash with PBS, the nitrocellulose-bottomed wells were exposed to a chromogen substrate solution consisting of 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP; Bio-Rad Lab. Richmond, CA) and *p*-nitroblue tetrazolium chloride (NBT; Bio-Rad) in 0·1 M $NaHCO_3$ plus 1 mM $MgCl_2$ pH 9·8. When spots reached maximal intensity, the reaction was stopped by thoroughly rinsing the plate with water. Spots were enumerated under a stereomicroscope with ×40 magnification.

For the enumeration of immunoglobulin-secreting cells, the wells were coated with $F(ab')_2$ fragments of goat anti-human IgA antibody (Pel-Freez, Rogers, AR) or $F(ab')_2$ fragments of goat anti-human IgG antibody (Jackson, West Grove, PA) or $F(ab')_2$ fragments of goat anti-human IgM antibody (Pel-Freez). The secondary antibodies were the same as in the antigen-specific assay.

ELISA

Serum, saliva, and peritoneal effluents were tested for TT-specific antibodies and total human IgA, IgA1, IgA2, IgG, and IgM in an ELISA. Polystyrene microtitre plates (Dynatech Lab., Alexandria, VA) were coated with 4 μg/ml of TT/ml overnight at 4°C for determination of TT-specific antibodies. After blocking with 1% bovine serum albumin (BSA), serial dilutions of the sample were incubated in the wells overnight at 4°C. Depending on the type of the assay, optimal dilutions of biotinylated goat anti-human IgA, IgG, IgM (Tago) or monoclonal anti-human IgA1 or IgA2 (Dr J. Radl and J. J. Haaijman, Rijswijk, The Netherlands) were added as secondary antibodies and incubated for 4 h at 37°C. The anti-IgA subclass MoAbs were detected with biotinylated goat anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, AL). The biotinylated antibodies were detected by incubating with 0·5 μg/ml avidin-horseradish peroxidase (Sigma) for 1 h at room temperature. Wells were then allowed to react with 2·5 mg/ml of the substrate 2,2 azinobis (3 ethyl-benzthiazoline sulphonic acid) (Sigma) in citrate buffer pH 4·2 containing 0·0075% H_2O_2. Tetanus hyperimmune globulin (Miles, Inc., Elkhart, IN) containing 250 U/ml of (mostly IgG) anti-TT antibody was used as the standard in the TT-specific IgG assay. In the ELISA for TT-specific IgA, IgA subclasses and IgM, reference sera were used which were assigned 250 ELISA units (EU)/ml of anti-TT of the respective isotype. Salivary anti-TT antibody levels were corrected for the influence of salivary flow rate by dividing the antibody level by the total level of the corresponding isotype [25]. The same principle was applied to peritoneal effluent antibody levels, which were divided by the total levels of the corresponding isotypes to correct for the variable dilution of peritoneal immunoglobulin by the dialysis fluid which is dependent on dwell time and the concentration of glucose and other osmotic substances in the dialysate.

In the ELISA for total immunoglobulin, the plates were coated with 2·5 μg/ml of $F(ab')_2$ fragments of goat anti-human IgA (Pel-Freez), 1·25 μg/ml of $F(ab')_2$ fragments of goat anti-human IgG (Jackson), 1 μg/ml of a mouse MoAb to human IgM (Hybridoma Core Facility at UAB), or 5 μg/ml of anti-human IgA1 or IgA2 MoAb (Dr J. Radl and J. J. Haaijman, Rijswijk, The Netherlands), dependent on the isotype to be assayed. Biotinylated goat anti-human IgA (for IgA, IgA1 and IgA2), IgG, and IgM (Tago), respectively, were used as secondary antibodies. A serum pool with known concentrations of isotypes served as a standard for total IgA, IgG, and IgM. IgA1κ (Kni) and IgA2λ (Fel) myeloma proteins were used as standard for IgA subclasses. Standard curves were constructed using a computer program based on the 4-parameter logistic model (Delta Soft, BioMetallics, Inc., Princeton, NJ).

Fluorescence-activated cell sorter (FACS)

Cell samples were stained for lymphocyte markers with PE-conjugated anti-CD3, PE-anti-CD4, FITC-conjugated anti-CD8, PE-anti-CD19 (all Becton Dickinson) MoAbs. CD5 expression was detected by staining with biotinylated anti-CD5 MoAb (Becton Dickinson) and avidin-PE/TXRD (a tandem of PE with Texas Red, Southern Biotechnology Associates). NK cells were identified with HNK-1 MoAb (kindly provided by Dr

Marianne Egan, UAB) plus biotinylated goat anti-mouse IgM and avidin-PE/TXRD (both Southern Biotechnology Associates). The cells were then subjected to three-colour analysis by using a FACStar (Becton Dickinson) equipped with an argon laser. Forward and side scatter were examined to denote cell size and granularity, respectively.

Cytoplasmic staining

Cells were stained for cytoplasmic immunoglobulin with FITC-labelled goat anti-human IgA, IgG, IgM, and TRITC-labelled anti-human immunoglobulin antibody [28,29]. The presence of cytoplasmic J chain was demonstrated by staining with TRITC-labelled anti-J chain antibody as previously described [29].

Statistical analysis

The results were expressed as arithmetic means with the s.e.m. The ANOVA with repeated measurements was used to determine significant differences among the two immunization routes (i.p. *versus* i.m.), significant changes of AbSC frequencies and antibody levels after immunization. A significance level of 0·05 was chosen in all statistical tests.

RESULTS

Safety of the i.p. administration of TT

All five individuals tolerated the i.p. immunization well. No apparent hypersensitivity reactions were observed. Two patients developed slightly cloudy peritoneal effluents 1–2 days after i.p. immunization. They showed no clinical signs of peritonitis such as fever or abdominal pain. Examination of the peritoneal fluid demonstrated no microorganisms on Gram stain and culture, although one patient had an increase of neutrophils to 74% on the differential count. The patients received prophylactic antibiotics. All four patients in the i.m. group tolerated the immunizations well.

Phenotype of peritoneal cells

Each peritoneal effluent (1·5–2·0 *l* of overnight dialysate, $n = 36$) yielded 0·5–10 $\times 10^6$ cells. Most peritoneal cells were lymphocytes (range 8–90%, mean 37%) followed by monocyte/macrophages (range 9–80%, mean 28%), polymorphonuclear leucocytes (range 0–74%, mean 4%) and mesothelial cells (range 0–18%, mean 2·5%). $CD3^+$ T cells represented the largest fraction (45–90%), while $CD19^+$ B cells accounted for 1–12% of all lymphocytes. There was a small percentage of HNK-1^+ NK cells among the lymphocytic elements. Figure 1a, b shows the phenotypic profile of peripheral blood and peritoneal lymphocytes from two patients collected and analysed simultaneously. Virtually all T cells were $CD5^+$, whereas up to 1/3 of all peritoneal B cells expressed CD5. Most $CD19^+$ cells bore surface IgA, IgG or IgM, which is characteristic for mature B cells. The phenotype of peritoneal B cells after immunization with TT was not examined.

Cytoplasmic immunoglobulin

By immunofluorescence, 0·4–1·7% of all lymphocytic elements were positive for intracytoplasmic immunoglobulin. The isotype distribution was 40–83% IgA, 17–45% IgG, and 0–20% IgM. Up to 34% of all immunoglobulin-positive cells were positive for J chain.

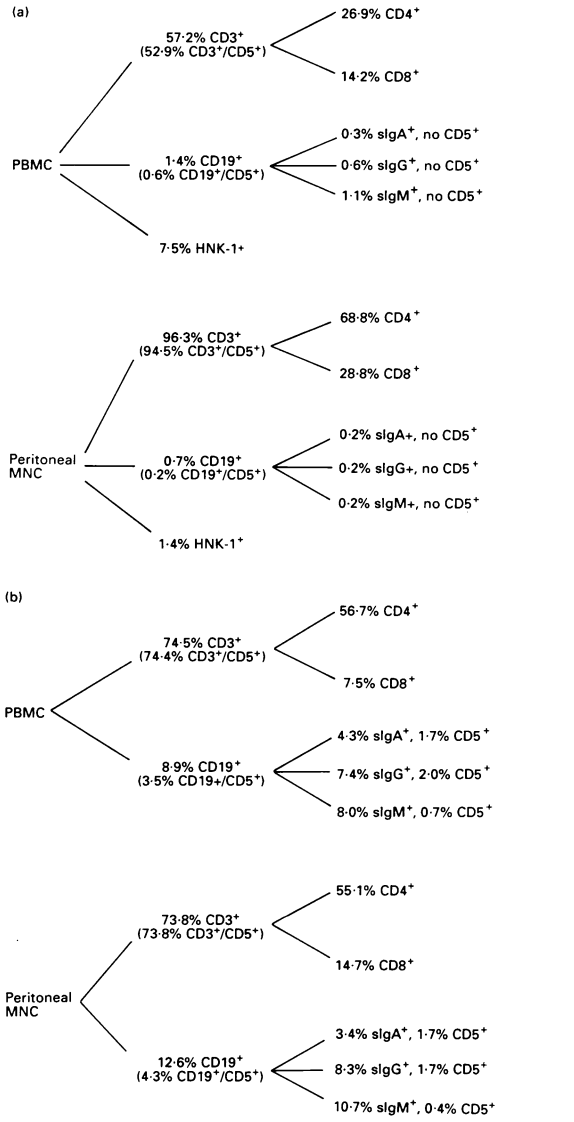

Fig. 1. Cell phenotype of peripheral blood and peritoneal mononuclear cells from two patients.

TT-specific AbSC

Before immunization, marginal numbers of AbSC were detectable by ELISPOT in the peripheral blood and peritoneal effluents of most patients. Seven days after i.p. or i.m. immunization with TT, significant numbers (ranging from 20 to 300/10^6 cells) of antigen-specific AbSC were found in the circulation and peritoneal effluents. This AbSC response was significant both in peripheral blood (P value for comparison of postimmune AbSC frequencies to preimmune values, IgA AbSC $P = 0{\cdot}025$, IgG AbSC $P = 0{\cdot}012$) and peritoneal cavity (IgA AbSC $P = 0{\cdot}022$, IgG AbSC $P = 0{\cdot}008$). IgG was the predominant isotype, while IgA accounted for virtually all of the remainder (Fig. 2). IgM anti-TT AbSC were found only occasionally. The frequency of AbSC declined when measured 14 and 21 days after immunization. Statistical comparison of AbSC frequencies in peripheral blood revealed no significant difference between the two routes, although i.m. immunization tended to induce high numbers of AbSC in the circulation. In contrast, i.p. immunization elicited significantly higher numbers of AbSC of the IgA and IgG

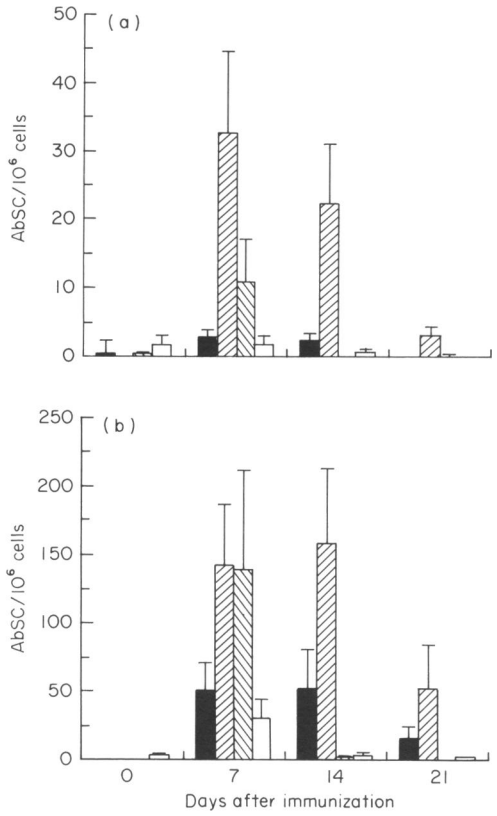

Fig. 2. IgA (a) and IgG (b) anti-tetanus toxoid (TT) antibody-secreting cells (AbSC) in peripheral blood and peritoneal cavity after i.m. or i.p. immunization with TT. Results represent means ± s.e.m. of four (i.m.) and five (i.p.) subjects, respectively. ■, Blood (i.p.); ▨, peritoneum (i.p.); ▧, blood (i.m.); □, peritoneum (i.m.).

isotype in peritoneal effluents than the i.m. route (IgA AbSC $P=0{\cdot}015$, IgG AbSC $P=0{\cdot}028$). Of interest was the observation that in several patients peritoneal AbSC remained detectable longer than peripheral blood AbSC.

Total IgSC

Peritoneal effluents were also analysed for total IgSC to determine whether immunization with TT by the i.p. or i.m. route induced polyclonal B cell activation. There were 27–170 IgASC per 10^6 cells with a subclass distribution of 50–87% IgA1 and 13–50% IgA2. IgG dominated with 180–440 IgGSC per 10^6 cells, whereas IgMSC were in the minority with 5–68 per 10^6 cells. Results suggested that there was no significant polyclonal B cell activation after immunization with TT. The isotype distribution in peripheral blood was slightly dominant for IgASC, although it was statistically significant only on day 7 after immunization in the i.p. group ($P=0{\cdot}043$).

TT-specific antibody in serum, saliva and peritoneal effluent

IgG and lower titres of IgA anti-TT antibody activity were measurable by ELISA in serum, saliva and peritoneal effluent of all individuals. Immunization with TT by either route elicited significant increases of IgG and IgA1 anti-TT antibody in serum and peritoneal effluents (Figs 3 and 4). IgA2 and IgM anti-TT antibody activities were either undetectable or only marginal in the ELISA. Salivary IgG anti-TT antibody levels increased significantly (Fig. 4), whereas salivary IgA did not. There were no significant differences in comparison of the two routes of immunization.

When antibody responses were expressed as fold-increases (FI), IgG constituted higher FI (geometric mean FI: 11·4 (i.p.) and 12·9 (i.m.)) than IgA (geometric mean FI: 5·6 (i.p.) and 7·4 (i.m.)) in serum. In saliva, FI for IgG were lower than in serum (geometric mean FI: 3·3 (i.p.) and 3·2 (i.m.)), whereas IgA antibody levels did not change significantly. In peritoneal effluents, FI for IgA and IgG were comparable (geometric mean FI in the 16–24 range).

Total immunoglobulin levels

The mean serum levels were 2550 ± 430 μg/ml (arithmetic mean ± s.e.m.) for IgA, 3677 ± 339 μg/ml for IgG and 2437 ± 687 for IgM. In saliva the levels were 336 ± 88 μg/ml and $11 \pm 2{\cdot}5$ μg/ml for IgA and IgG, respectively. The levels in peritoneal effluents were $8{\cdot}9 \pm 2{\cdot}1$ μg/ml, 66 ± 25 μg/ml, $0{\cdot}37 \pm 0{\cdot}13$ μg/ml for IgA, IgG and IgM, respectively. There was no significant difference between the two routes of immunization with respect to the immunoglobulin levels in serum and saliva, and no significant changes occurred after immunization.

DISCUSSION

The peritoneal route of immunization has been used in experimental animals as probably the most effective site of antigen deposition for induction of both systemic and mucosal immune responses [8–10]. The discovery of peritoneal B1 cells representing a major source for intestinal IgA plasma cells in mice [11] prompted us to study the potential of human peritoneal B cells to differentiate into AbSC. This is, to our knowledge, the first report of an artificial i.p. immunization in human subjects. Our study agrees with the findings by several other investigators [16–19] that peritoneal effluents from patients on CAPD contain sizable numbers of lymphocytes and macrophages. A closer examination of the peritoneal B lymphocyte portion showed that the cells bore surface IgA, IgG or IgM as previously reported [20]. A smaller fraction of B cells expressed intracytoplasmic immunoglobulin and J chain. It has been proposed that the presence of intracellular J chain is indicative of the clonal immaturity of B cells expressing various immunoglobulin isotypes, including IgG and IgD, and J chain-positive cells are found especially in mucosal tissues [30]. We found by FACS analysis that up to 1/3 of peritoneal B cells expressed CD5. In addition to the phenotypic characterizations, we demonstrated that peritoneal B cells actively secrete IgA, IgG and IgM. The i.p. administration of the purified protein antigen TT induced the appearance of antigen-specific AbSC in the peritoneal cavity and the circulation, as well as significant antibody responses in serum, saliva and peritoneal effluents. In comparison with the control group, the i.p. route of immunization elicited higher numbers of peritoneal AbSC. Peripheral blood AbSC frequencies and antibody responses in serum, saliva and peritoneal effluents did not show significant differences among the two routes of immunization.

Analysis of the different components of the immune response suggests that i.p. immunization led to the local response of resident B cells, as manifested by the appearance of specific AbSC, and also to a systemic response after drainage of the antigen via lymphatics into the circulation. Alternatively, TT-

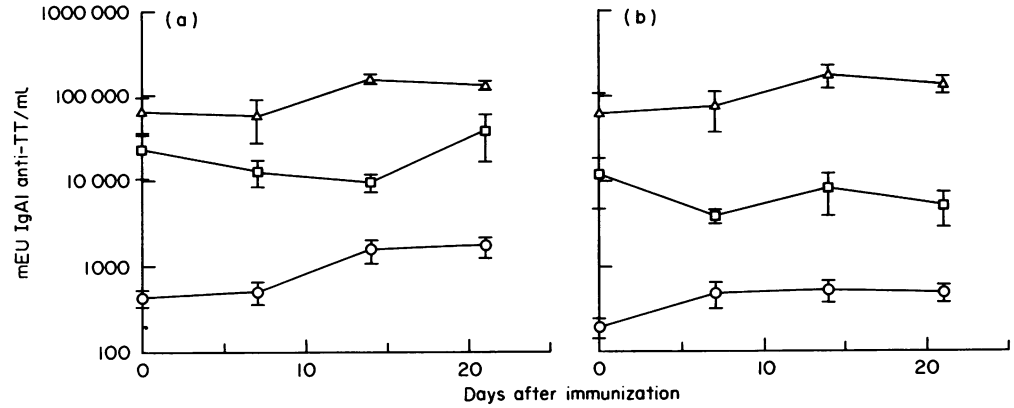

Fig. 3. IgA1 anti-tetanus toxoid (TT) antibody levels in serum, saliva, and peritoneal dialysates after i.p. (a) and i.m. (b) immunization with TT. Results are shown as means ± s.e.m. of four (i.m.) and five (i.p.) subjects, respectively. ○, Peritoneal fluid; □, saliva; △, serum.

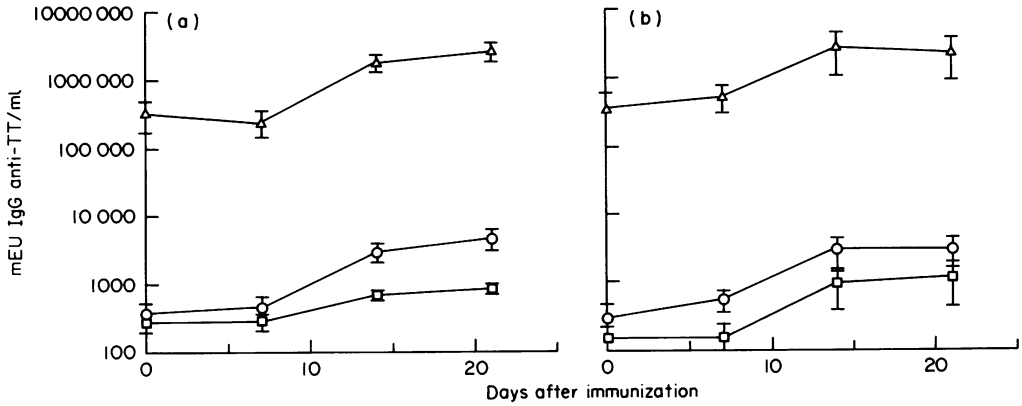

Fig. 4. IgG anti-tetanus toxoid (TT) antibody levels in serum, saliva, and peritoneal dialysates after i.p. (a) and i.m. (b) immunization with TT. Results are shown as means ± s.e.m. of four (i.m.) and five (i.p.) subjects, respectively. ○, Peritoneal fluid; □, saliva; △, serum.

stimulated lymphoblastoid B cells could have left the peritoneal cavity via the thoracic duct to enter the circulation, where they served as vehicles for the systemic dissemination of the immune response, as previously shown in an animal model [31]. Anti-TT antibodies in the peritoneal effluent were probably derived from the circulation, as previous studies have shown the transfer of serum IgA and IgG into the peritoneal fluid [32,33]. It is also conceivable that AbSC in the peritoneal cavity contributed to anti-TT antibody found in the peritoneal effluents. Immunization with TT did not induce polyclonal B cell activation in the peritoneal cavity, which would have accounted for a non-specific increase in the number of anti-TT AbSC. The finding that IgASC dominated in peripheral blood is consistent with previous studies [23,24,34].

IgG anti-TT antibody is the only isotype that showed a significant increase in saliva after either route of immunization. That increase is probably not due to local production in mucosal tissues, but secondary to passive transmucosal transfer of serum-derived IgG [24,35,36]. Serum IgA, on the other hand, did not contribute to the salivary IgA pool, a feature distinctive of the separation of the serum and secretory IgA systems [37,38]. The i.m. immunization induced also specific AbSC in peritoneal effluents, although at lower frequencies than the i.p. route. The AbSC were probably derived from the circulation through migration to milky spots via omental stomata [7]. It is less likely the TT administered intramuscularly could have reached the peritoneal cavity to trigger resident B cells. The fact that i.p. immunization did not elicit a salivary IgA response does not support the hypothesis that human peritoneal B cells serve as precursors of salivary IgA plasma cells as suggested in the murine model [11,12]. However, we have not performed experiments involving a local challenge with TT injected into a remote mucosal surface. This challenge may be necessary to stimulate a secretory IgA response after parenteral immunization [39]. Moreover, intestinal and salivary IgA responses do not correlate, thus the lack of a salivary response does not equal lack of intestinal response. Of note is Kroese's observation that most murine peritoneal B cell precursors were surface immunoglobulin-negative, thus lacking the antigen receptor [11]. All patients had received previous immunizations with TT. Therefore, our administration of TT represented a booster immunization, the response to which is primarily IgG rather than IgA [23]. Antigens that have been shown to elicit primarily IgA responses such as bacterial polysaccharides may have a more stimulating effect on the presumptive IgA precursors in the peritoneal cavity.

The fact that patients with chronic renal failure were studied here represents a limitation, because immunological abnormalities have been described in that population of patients [40–42]. The immune response to hepatitis B virus vaccine is diminished

in patients with chronic renal failure. However, more recent studies showed virtually normal immune responses to several common vaccines [41,43–45]. The numbers of peripheral blood B and T cells from uraemic patients were within normal range, and B cells responded normally to Epstein–Barr virus (EBV) and pokeweed mitogen (PWM) *in vitro* with secretion of immunoglobulin [46]. The lymphocyte responses to phytohaemagglutinin (PHA) and *Staphylococcus aureus* strain Cowan were abnormally low. A metabolic monocyte defect rather than an intrinsic B cell abnormality appears to be responsible for the diminished immune responses to hepatitis B vaccination [40,47]. Interestingly, patients on CAPD appeared to be less affected by that immune abnormality than patients on haemodialysis [42]. However, peritoneal dialysis, itself, could have an effect on the mucosal and systemic immune systems, e.g. through continuous removal of immune-competent cells and specific antibody.

Taken together, this study demonstrates that i.p. administration of a purified protein antigen elicits a response of peritoneal AbSC as well as a systemic antibody response. The absence of a significant secretory IgA response makes the peritoneal cavity less likely to be an inductive site for secretory IgA. However, the peritoneal cavity may play a role in the common mucosal immune system as a reservoir for precursors of cells that migrate to the mucosa-associated lymphoid tissues before their antigen-dependent differentiation to AbSC.

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