Kidney International, Vol. 46 (1994), pp. 443–454

Immunohistochemical studies of the peritoneal membrane and infiltrating cells in normal subjects and in patients on CAPD

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Immunohistochemical studies on the peritoneal membrane and infiltrating cells in normal subjects and in patients on CAPD. We performed immunohistochemical studies on biopsies of the parietal peritoneal membrane of 33 subjects to investigate whether other cell populations, in addition to mononuclear cells free in the dialysate, might participate in the defense of the peritoneum against microbial invasion during CAPD. Leukocytes were found to concentrate in two areas: a submesothelial layer composed of elongated macrophages displaying activation and maturation markers, and perivascular, less mature macrophages closely associated with T cells and HLA-DR, ICAM-1 and VCAM-1 expressing endothelial cells. Normal mesothelial cells were found to express constitutively the transferrin receptor and the adhesion molecules ICAM-1 and VCAM-1 but not ELAM-1. There were no major differences between normal and uremic subjects, while peritoneal dialysis patients exhibited minor derangements of the submesothelial layer and slight up-regulation of the expression of HLA-DR on endothelial cells. Peritonitis was associated with increased submesothelial cellularity and, particularly, perivascular leukocyte infiltration accompanied by increased expression of HLA-DR and adhesion molecules. Besides mononuclear cells free in the dialysate, this study demonstrates the existence of two additional peritoneal membrane leukocyte populations: submesothelial macrophages, and perivascular macrophages and T cells. It also suggests the existence of a fourth population of intracavitary leukocytes adherent to mesothelial cells. Studies are now necessary to evaluate their exact role in the host defence against peritonitis during CAPD.

Fifteen years after its introduction, continuous ambulatory peritoneal dialysis (CAPD) is now used world-wide for the treatment of patients with end-stage renal disease [1]. Familiarity with the method and technical developments have lessened, but not eliminated, episodes of contamination of the peritoneal cavity resulting in clinical peritonitis and associated morbidity [2–4].

Phagocytosis performed by macrophages is believed to represent one of the main defenses of the peritoneal cavity against invading pathogens [5–7]. Evidence from a rat model [8] indicates that peritoneal defense mechanisms suffice to clear invading bacteria below a threshold level of contamination.

The CAPD technique interferes with these mechanisms in a number of ways. Firstly, the peritoneal access facilitates the ingress of micro-organisms into the peritoneal cavity [9]. Also, *in vitro* studies have shown that the addition of fresh dialysis fluid adversely affects the function of resident peritoneal phagocytes [10] and may dilute opsonins and phagocytic cells below the concentration required for efficient phagocytosis [5, 11, 12]. Despite these observations cells in spent dialysis fluid are easily obtained and have been the major target of studies aiming at the delineation of the mechanisms of peritoneal defense [7].

It has been suggested recently that phagocytes "floating" free in the dialysis fluid may not be representative of those responsible for the defense of the peritoneal cavity in view of the low probabilities of random interaction with invading bacteria [7, 13]. Rather, other cell populations such as those adherent to the mesothelium or located within the peritoneal membrane and its interstitium may be more important.

The present study was designed to investigate the distribution of immune cells and of adhesion molecules participating in cell-leukocyte interactions on both endothelial and mesothelial cells in the peritoneal membrane. Our results show that the peritoneal interstitial tissue displays an arrangement of cells that places submesothelial macrophages in a privileged location to meet invading micro-organisms. We found also that peritoneal membrane blood vessels are surrounded by closely associated macrophages and T cells suggesting engagement in activities such as antigen presentation and/or cell activation. Moreover, besides endothelial cells, mesothelial cells express adhesion molecules *in vivo* that can provide accessory support for phagocyte and lymphocyte activities within the peritoneal cavity. We further investigated the distribution of leukocytes, their expression of activation markers and of adhesion molecules on endothelial and mesothelial cell in patients on CAPD with and without peritonitis.

Methods

Patients

We studied a total of 33 individuals (Table 1): seven normal controls, nine uremic control patients with ESRD prior to initiation of peritoneal dialysis, eight on stable CAPD without clinical evidence of peritonitis, and nine with CAPD-associated peritonitis. Biopsies from this last group were taken at the moment of catheter removal following failure of the initial antibiotic regime—in all cases composed of vancomycin and a third generation cephalosporin.

Received for publication September 8, 1993
and in revised form February 24, 1994
Accepted for publication February 28, 1994

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Table 1. Clinical data

Patients	Age *years*	Number of episodes of peritonitis	Time since last episode of peritonitis	Time on CAPD *months*	Renal disease
Normal controls (N = 7)					
1 to 7	range (27–70)	—	—	—	—
Mean ± SD	52.7 ± 16.2				
Uremic controls (N = 9)					
8	71	—	—	—	IgA disease
9	59	—	—	—	Polycystic disease
10	60	—	—	—	Obstructive uropathy
11	22	—	—	—	Mesangiocapillary GN
12	48	—	—	—	Unknown etiology
13	53	—	—	—	Reflux nephropathy
14	45	—	—	—	Primary amyloidosis
15	61	—	—	—	Nephrolithiasis
16	36	—	—	—	Hypertensive nephrosclerosis
Mean ± SD	50.6 ± 14.8				
CAPD patients (N = 8)					
17	68	1	12 m	18	Secondary amyloidosis
18	40	2	14 m	25	Chronic GN
19	60	0	—	10	LCDD
20	68	1	3 m	7	Polycystic disease
21	35	4	9 m	39	Chronic GN
22	64	2	7 m	11	Membranous nephropathy
23	35	2	14 m	39	Chronic GN
24	45	2	4 m	29	Polycistic disease
Mean ± SD	51.9 ± 14.6				
Peritonitis patients (N = 9)					
25	72	3	3 d	52	Hypertensive nephrosclerosis
26	67	1	4 d	53	Obstructive uropathy
27	24	5	12 d	48	Chronic GN
28	57	2	5 d	30	Crescentic GN
29	64	1	5 d	62	Unknown etiology
30	76	1	12 d	15	Diabetic nephropathy
31	28	3	4 d	13	Mesangiocapillary GN
32	72	0	8 d	3	Chronic GN
33	46	0	14 d	3	Hypertensive nephrosclerosis
Mean ± SD	56.2 ± 19.4				

Abbreviations are: SD, standard deviation; m, months; d, days; GN, glomerulonephritis; LCDD, light chain deposition disease.

Source of material

Biopsies of the parietal peritoneum were obtained from the borders of anterior abdominal wall incisions during elective or catheter-related procedures, from small hernia sacs, and from the pelvic peritoneum during renal transplantation.

The peritoneum was carefully approached from the antemesothelial side. The sample was quickly isolated with a curved surgical clamp and cut. We tried to avoid undue manipulation, particularly extended exposition and surgical handling, which usually causes tissue disruption [14]. As an extra measure any specimens with suspected sampling artifacts such as leukocyte clogging were discarded. Care was also taken to avoid biopsying the peritoneum around current or previous catheter entry sites.

Fragments were cut in sheets of 0.5 to 1.0 cm, oriented, embedded in OCT compound, immediately frozen in liquid nitrogen and stored at −70°C until processing. To allow sectioning of some very thin membranes, peritoneal sheets were sometimes folded in two so that the mesothelial layers would face each other. Once again, all handling was done with special caution to avoid harm to the tissue and denudation of mesothelial cells.

Immunohistology

Glass slides were treated with an alcoholic solution of 3-aminopropyltriethoxysilane to facilitate adhesion of the fragile tissue fragments [15], without which most of the specimens floated off. Serial vertical cryostat sections were cut at 6 to 10 μm (thinner sections were difficult to obtain due to the loose structure of the membrane) and fixed in acetone at 4°C for 10 minutes. Consecutive sections were prepared to investigate the expression of multiple markers in the same cell population. At least two (usually 3) sections were stained with each antibody.

The indirect immunoperoxidase technique used in this study has been described previously [16]. In brief, sections were sequentially incubated with 1/5 normal rabbit serum for 10 minutes, monoclonal antibodies (MoAbs) at appropriate dilutions (Table 2) for 90 minutes, and peroxidase-conjugated rabbit anti-mouse IgG for 30 minutes. After the last two steps the sections were extensively washed in phosphate-buffered saline (PBS). Slides were then treated with 3′5′diaminobenzidine for approximately two minutes, counter stained with Meyer's Haemalum, and mounted in a permanent medium. In each staining session we processed a negative control with substitution of PBS for the primary monoclonal antibody, and positive controls for each antibody (tonsil sections).

Study of different cell populations

Analysis was done without information about the patient identity or clinical condition. Cells were considered positive when

Table 2. Monoclonal antibodies employed with respective dilutions and characteristic specificities

Antibody	Specificity	Phenotypes	Dilutions	Source
FMC32	Monocytes; MØ; DC	CD 14	1:40	Serotec
Leu M3	Monocytes; MØ; DC	CD 14	1:20	Becton Dickinson
RFD7	Mature tissue MØ	Group 12	1:40	Royal Free Hospital
Pan-T cocktail	T cells	CD 2 + 7 + 8 + 27	1:10	Royal Free Hospital
UCHT 1	T cells	CD 3	1:40	Dakopatts
To15	B cells	CD 22	1:40	Dakopatts
Leu7	Natural killer cells	CD 56	1:40	Becton Dickinson
DK22	HLA-DR	—	1:20	Dakopatts
TFR	Transferrin receptor	CD 71	1:10	Becton Dickinson
To5	CR1 (C3b receptor)	CD 35	1:20	Dakopatts
ACT-1	IL-2 receptor	CD 25	1:10	Dakopatts
6-5b5	ICAM-1	CD 54	Neat	Dr. D. Haskard
1-2b6	ELAM-1	--	Neat	Dr. D. Haskard
1-4c3	VCAM-1	—	Neat	Dr. D. Haskard
Pal-E	Venules and capillaries	—	1:20	Sanbio
Ki-67	Proliferating cells	—	1:20	Dakopatts

Abbreviations are: MØ, macrophages; DC, dendritic cells.

a continuously stained plasma membrane surrounding a clearly visible nucleus was identified. Based on preliminary studies leukocytes were studied in the interstitium underneath the mesothelial basement membrane (submesothelial area) and surrounding peritoneal venules and capillaries (perivascular area). Their spatial distribution was noted and the number of positive cells in each area was estimated in separate and expressed according to the following semi-quantitative scale: (0) negative; (1) rare positive cells; (2) even distribution of small numbers and/or small clusters of positive cells; (3) diffuse distribution of positive cells comprising more than 50% of visible nuclei; and (4) positive cells forming focal or diffuse inflammatory infiltrates.

The invariable uniform staining of venular and capillary endothelium produced by the monoclonal antibody Pal-E was used as the standard to which the staining produced by the other antibodies was compared. On both endothelial and mesothelial cells, we employed the following scale: (0) negative; (1) faint; (2) less intense than Pal-E; (3) intensity equal to Pal-E; and (4) more intense than Pal-E.

After the semi-quantitative scores were obtained, biopsies were once again analyzed, this time to study the staining for the different markers on sequential sections.

Statistical analysis

The median, mean and standard deviation of the semiquantitative scores were calculated for each group of patients. The differences in the scores among the groups were analyzed with the Kruskall-Wallis non-parametric test. Values of $P < 0.05$ were considered significant. In these cases, differences between individual groups were ascertained by performing pairwise comparisons with the Mann-Whitney U test and the level of significance adjusted with the Bonferroni procedure. Since the two controls groups were considered as a single one (see below), the protection level was set at 0.05/3 or $P < 0.017$. The statistical analysis was performed on a MacIntosh personal computer using the Systat package (Systat, Inc., Evanston, Illinois, USA).

Results

Immunohistology of the peritoneal membrane in normal control individuals and in non-dialyzed uremic patients

General architecture and distribution of cells. The general architecture and cell distribution of biopsies from normal individuals did not differ from that of patients with renal failure prior to initiation of peritoneal dialysis. The distribution of leukocytes, the antigens they expressed, as well as the antigens expressed by endothelial and mesothelial cells were also similar (Tables 3, 4, 5 and 6), and did not reach statistical significance in any of phenotypic parameters evaluated (Mann-Whitney U test). The description that follows (and all pairwise comparisons) apply to the immunohistology of both groups considered as a single control group.

The distribution of immune cells in the peritoneal tissue can be seen in Figure 1a. Mesothelial cells formed a single layer of flattened or ovoid cells with large ovoid nuclei underneath which run a continuous basement membrane. Beneath and parallel to the membrane, interstitial cells were arranged in rows separated by bundles of connective tissue that comprised most of the interstitium. Usually a short distance into the membrane lay a capillary plexus, and often deeper in, a vascular network composed of capillaries, venules and arterioles. Interstitial cells in these areas were distributed in a concentric fashion of one or more loose layers of cells surrounding the blood vessels.

The submesothelial interstitium.

Macrophages. Irregularly shaped cells bearing macrophage phenotypes, elongated and rich in cytoplasmic extensions, were arranged in a peculiar submesothelial array. While in the deep layers of the membrane, they followed the general pattern of cells interspersed with connective tissue fibers; in the first layer close to the peritoneal cavity, they formed an almost continuous sheet of phagocytes apposed to the basement membrane. Macrophages in this area had abundant cytoplasm spread sideways like a "fried egg" compressed between sheets of connective tissue (Fig. 1 a and b). These cells were usually HLA-DR and RFD7 positive. Many also expressed ICAM-1, the complement receptor type 1 (CR1) and, to a lesser extent, the transferrin receptor (TFR).

Lymphoid cells. Cells with B-lymphocyte (CD22) or NK cell (CD56) phenotypes were not usually seen in the submesothelial interstitium. T cells were scant in layers close to the peritoneal cavity although single T cells could be found in small numbers scattered in the deeper layers. Their pattern of distribution was clearly different from the macrophages that concentrated immediately under the mesothelial layer. Since HLA-DR and TFR were also found on macrophages, it was impossible with the methods used in this study to be sure whether these markers were

Table 3. Phenotypes and activation markers on submesothelial cells in peritoneal biopsies

Phenotypes & other markers		Normal controls ($N = 7$)	Uremic controls ($N = 9$)	CAPD patients ($N = 8$)	Peritonitis patients ($N = 9$)	P^a
MØ (FMC 32)	median	2.0	2.0	2.0	4.0[dc]	0.011
	mean ± SD	1.86 ± 0.90	2.0 ± 0.71	1.87 ± 0.99	3.33 ± 0.87	
MØ (Leu M3)	median	2.0	1.0	1.0	2.0	NS
	mean ± SD	1.71 ± 0.76	1.44 ± 0.73	1.62 ± 1.06	2.33 ± 1.32	
MØ (RFD7)	median	1.0	1.0	1.0	2.0	NS
	mean ± SD	1.43 ± 0.79	1.56 ± 0.88	1.14 ± 1.07	1.78 ± 0.97	
T cells (Pan T)	median	1.0	1.0[c]	1.0	1.0[b]	NS
	mean ± SD	0.86 ± 0.69	1.00 ± 0.58	0.75 ± 0.46	1.12 ± 0.35	
T cells (UCHT 1)	median	1.0	1.0	1.0	1.0	NS
	mean ± SD	0.86 ± 0.69	1.00 ± 0.50	0.62 ± 0.52	1.11 ± 0.33	
B cells (To 15)	median	0.0	0.0	0.0	0.0	NS
	mean ± SD	0.0	0.11 ± 0.33	0.0	0.11 ± 0.33	
NK cells (Leu 7)	median	0.0	0.0	0.0	0.0	NS
	mean ± SD	0.0	0.0	0.12 ± 0.35	0.11 ± 0.33	
DR	median	2.0	2.0	2.0	4.0[de]	0.008
	mean ± SD	2.14 ± 0.69	2.22 ± 0.44	1.87 ± 0.99	3.33 ± 0.87	
CR1	median	1.0	1.0	1.0	1.5[b]	NS
	mean ± SD	0.86 ± 0.64	0.89 ± 0.78	1.71 ± 1.13	1.62 ± 0.74	
TFR	median	1.0	1.0	0.0	1.0	NS
	mean ± SD	0.57 ± 0.53	0.67 ± 0.50	0.75 ± 1.39	1.67 ± 1.22	
IL-2R (TAC)	median	0.0	0.0	0.0	0.0	NS
	mean ± SD	0.0	0.0	0.0	0.22 ± 0.44	
ICAM-1	median	1.0	1.0[b]	1.0	2.0[de]	0.002
	mean ± SD	1.00 ± 0.00	1.12 ± 0.35	1.00 ± 0.535	2.22 ± 0.97	

Abbreviations are: MØ, macrophage; DR, HLA-DR; CR1, complement receptor 1; TFR, transferrin receptor; IL-2R, interleukin 2 receptor; NS = not significant.

[a] Kruskall-Wallis test used for statistics

[b] Material from one patient unavailable for analysis

[c] Material from two patients unavailable for analysis

[d] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to controls

[e] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to stable CAPD patients

Table 4. Phenotypes and activation markers on perivascular cells in peritoneal biopsies

Phenotypes & other markers		Normal controls ($N = 7$)	Uremic controls ($N = 9$)	CAPD patients ($N = 8$)	Peritonitis patients ($N = 9$)	P^a
MØ (FMC 32)	median	2.0	2.0	2.0	4.0[d]	0.002
	mean ± SD	1.86 ± 0.38	2.11 ± 0.33	2.25 ± 0.89	3.44 ± 0.88	
MØ (Leu M3)	median	2.0	2.0	2.0	3.0	NS
	mean ± SD	1.57 ± 0.53	1.67 ± 0.71	1.75 ± 0.71	2.67 ± 1.12	
MØ (RFD7)	median	2.0	1.0	1.0	2.0	NS
	mean ± SD	1.71 ± 0.49	1.33 ± 0.50	1.25 ± 0.46	1.89 ± 0.93	
T cells (Pan T)	median	2.0	2.0[c]	1.0	2.0[b]	NS
	mean ± SD	1.71 ± 0.49	1.71 ± 0.49	1.62 ± 1.06	2.37 ± 0.92	
T cells (UCHT 1)	median	2.0	1.0	1.0	2.0	NS
	mean ± SD	1.71 ± 0.49	1.44 ± 0.28	1.14 ± 1.07	2.11 ± 0.93	
B cells (To 15)	median	0.0	0.0	0.0	0.0	NS
	mean ± SD	0.0	0.0	0.12 ± 0.35	0.44 ± 0.88	
NK cells (Leu7)	median	0.0	0.0	0.0	0.0	NS
	mean ± SD	0.14 ± 0.38	0.0	0.25 ± 0.46	0.33 ± 0.50	
DR	median	2.0	2.0	2.0	4.0[de]	0.001
	mean ± SD	2.29 ± 0.49	2.11 ± 0.33	2.25 ± 0.89	3.67 ± 0.71	
CR1	median	1.0	1.0	1.0	1.0[b]	NS
	mean ± SD	0.71 ± 0.76	0.89 ± 0.60	1.12 ± 1.25	1.50 ± 0.53	
TFR	median	1.0	1.0	0.0	1.0[d]	0.04
	mean ± SD	0.71 ± 0.49	0.56 ± 0.53	0.50 ± 1.07	1.44 ± 0.88	
IL-2R (TAC)	median	0.0	0.0	0.0	0.0	NS
	mean ± SD	0.11 ± 0.38	0.0	0.0	0.33 ± 0.50	
ICAM-1	median	1.0	1.0[b]	1.0	3.0[d]	0.02
	mean ± SD	1.14 ± 0.38	0.87 ± 0.35	1.12 ± 1.25	2.56 ± 1.42	

Abbreviations are: MØ, macrophage; DR, HLA-DR; CR1, complement receptor 1; TFR, transferrin receptor; IL-2R, interleukin 2 receptor; NS = not significant.

[a] Kruskall-Wallis test used for statistics

[b] Material from one patient unavailable for analysis

[c] Material from two patients unavailable for analysis

[d] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to controls

[e] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to stable CAPD patients

Table 5. Activation markers and adhesion molecules on endothelial cells in peritoneal biopsies

Phenotypes & other markers		Normal controls ($N = 7$)	Uremic controls ($N = 9$)	CAPD patients ($N = 8$)	Peritonitis patients ($N = 9$)	P^a
DR	median	2.0	2.0	2.0	3.0[c]	0.02
	mean ± SD	1.86 ± 0.69	2.11 ± 0.60	2.25 ± 1.04	3.22 ± 0.83	
ICAM-1	median	2.0	2.0[b]	2.0	3.0[cd]	0.013
	mean ± SD	1.71 ± 0.76	1.62 ± 0.52	1.75 ± 0.46	2.67 ± 0.71	
VCAM-1	median	1.0	1.0[b]	1.0	1.0	NS
	mean ± SD	0.86 ± 0.69	0.75 ± 0.46	0.63 ± 0.52	1.78 ± 1.30	
ELAM-1	median	0.0	0.0[b]	0.0	1.0[c]	0.03
	mean ± SD	0.29 ± 0.49	0.12 ± 0.35	0.38 ± 0.74	1.0 ± 0.71	

Abbreviations are: DR, HLA-DR; NS, not significant.
[a] Kruskall-Wallis test used for statistics
[b] Material from one patient unavailable for analysis
[c] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to controls
[d] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to stable CAPD patients

Table 6. Proliferation markers and adhesion molecules on mesothelial cells in peritoneal biopsies

Phenotypes & other markers		Normal controls ($N = 7$)	Uremic controls ($N = 9$)	CAPD patients ($N = 8$)	Peritonitis patients ($N = 9$)	P^a
TFR	median	2.0	2.0	1.0[d]	1.0[b]	0.036
	mean ± SD	2.14 ± 0.69	2.11 ± 0.60	1.38 ± 0.52	1.43 ± 0.79	
Ki-67	median	0.0	0.0	0.0	0.0[b,c]	NS
	mean ± SD	0.0	0.0	0.0	0.29 ± 0.76	
ICAM-1	median	2.0	2.0	1.0[d]	1.0[b]	0.045
	mean ± SD	1.86 ± 0.53	2.00 ± 0.50	1.25 ± 0.46	1.29 ± 0.95	
VCAM-1	median	2.0	2.0	1.0	1.0[b]	NS
	mean ± SD	1.57 ± 0.53	1.56 ± 0.53	1.38 ± 0.52	1.57 ± 0.79	

Abbreviations are: TFR, transferrin receptor; NS, not significant.
[a] Kruskall-Wallis test used for statistics
[b] Material from two patients unavailable for analysis
[c] Staining observed in some of the cells on a multilayered mesothelial cell lining
[d] Mann-Whitney U test with Bonferroni correction, $P < 0.017$ when compared to controls

also expressed on isolated T cells. Except around one blood vessel from one patient, no other cells expressing the IL-2 receptor (IL-2R) were seen in biopsies from the two control groups. Similarly, not a single cell in the peritoneal interstitium of the two control groups expressed the Ki-67 marker of cell proliferation in any biopsy.

The perivascular interstitium. A group of HLA-DR expressing cells was always seen in close proximity to the peritoneal blood vessels. Although the peritoneal endothelium expressed class II antigens constitutively (see below) the staining of perivascular cells was usually more intense. These cells did not express the Ki-67 proliferation-associated antigen.

Macrophages and monocytes. Most of the cells surrounding capillaries, venules and arterioles expressed the CD14 phenotype and were HLA-DR positive (Fig. 2a). While many macrophages expressed the RFD7 antigen, the population seemed heterogeneous in this regard, as some cells apparently did not express it. Perivascular macrophages expressed CR1, TFR, and ICAM-1 with less intensity than HLA-DR. It is noteworthy that these cells were situated in close association with blood vessels and T cells (see below).

Lymphoid cells. Small numbers of T cells identified by the MoAb UCHT1 (anti-CD3) and the pan T cell cocktail (mixture of MoAbs anti-CD2, CD7, CD8, and CD27) were regularly found in a perivascular location, particularly around venules (Fig. 2b). Again, perivascular B cells and NK cells were practically not found.

Endothelial cells. The immunohistochemical staining was uniform for all endothelial cells on a given type of vessel. In general, for the antibodies used in this study, arterioles showed less staining than venules and capillaries. Endothelial cells constitutively presented moderate expression of HLA-DR and ICAM-1. The staining was heavier in venules and capillaries closer to the mesothelium than in arterioles. The adhesion molecule VCAM-1 was expressed with less intensity while the expression of ELAM-1 was faint, if at all present.

Mesothelial cells. The immunochemical staining of mesothelial cells was generally uniform; that is, all cells in the preparation were either positive or negative. Mesothelial cells characteristically expressed the adhesion molecules ICAM-1, VCAM-1 and the transferrin receptor (Fig. 3 a, b, c). ELAM-1, HLA-DR, Ki-67 or any of the other markers were not detected.

CAPD patients

The submesothelial area. In patients on CAPD without clinical evidence of peritonitis, the continuous line of phagocytes under the basement membrane was less evident. Also there was usually some degree of interstitial disorganization and edema. Probably for this reason biopsies were more difficult to cut. However, in most patients the pattern was similar to that of controls, and the

semi-quantitative scores for macrophages and other markers did not differ (Table 3), despite the impression that the cells were apparently fewer and more dispersed within the interstitial matrix.

The perivascular area. There was no major change in the distribution of lymphoid cells (Table 4). There was also no statistically significant change in the perivascular macrophage population (Table 4). However, there was a trend, supported by the examination of consecutive slides, that a higher proportion of immature macrophages (without RFD7 antigen expression) was present. The expression of HLA-DR, ICAM-1, CR1 and TFR was maintained (Table 4) although the intensity was also apparently slightly less than that of the non-dialyzed peritoneum. Once again these differences were not statistically significant. In one patient (patient 20) we identified a superficial pericapillary infiltrate of RFD7-negative macrophages with marked expression of HLA-DR that disrupted the basement membrane and erupted into the peritoneal cavity. In another area of the same biopsy there was a dense vascular and perivascular infiltrate composed exclusively of T cells (Fig. 4). This patient had her first episode of peritonitis three months before the biopsy was taken.

Endothelial cells. Endothelial cells in the peritoneum of CAPD patients did not show major changes in the expression of ICAM-1 and VCAM-1 (Table 5). The expression of HLA-DR on the superficial capillary plexus was increased in three cases, particularly in the patient with a pericapillary infiltrate. This patient also had moderate staining for ELAM-1 on the capillaries closer to the membrane.

Mesothelial cells. The mesothelial expression of ICAM-1 and VCAM-1 was unchanged in CAPD patients. However, the staining for TFR was significantly lighter in comparison with the two groups of undialyzed patients (Table 6).

CAPD patients with peritonitis

The submesothelial area. As expected, biopsies of patients with peritonitis showed an increased number of macrophages (Table 3) either diffusely or as focal submesothelial infiltrates. Although the actual semi-quantitative score did not change, a greater proportion of the cells were immature macrophage (RFD7 negative) as the numbers of macrophages expressing the CD14 antigen had increased significantly. Increased expression of HLA-DR and ICAM-1 was also seen in association with peritonitis. In two patients the interstitial infiltrates included cells with high endogenous peroxidase content, possibly polymorphonuclear leukocytes. The pattern of lymphoid cells distribution in this area did not differ from the other groups. In particular there was no infiltration of T cells in patients with peritonitis.

The perivascular area. Seven out of the nine patients studied had marked perivascular leukocyte infiltrates. The cell content of these infiltrates was heterogeneous with a predominance of macrophages (Fig. 5a), most of them lacking the RFD7 antigen (Fig. 5b). The majority of these cells displayed heavy expression of HLA-DR (Fig. 5c) and moderate expression of ICAM-1. The expression of CR1 and TFR was also increased but only the latter reached statistical significance.

The perivascular infiltrates also included T cells (Fig. 5d), a very small proportion of which expressed the IL-2 receptor. In one patient (patient 28) the infiltrate had the characteristics of a true arteriolitis that included macrophages, activated T cells, B cells and a small number of NK cells. It is interesting, and possibly relevant, that this patient had a crescentic glomerulonephritis diagnosed 14 years ago, and had spent a decade living with a renal transplant and on renal replacement therapy without evidence of active vasculitis.

Endothelial cells. Wherever perivascular infiltrates were present a striking up-regulation on the expression of HLA-DR occurred on endothelial cells (Fig. 5c). Endothelial cells also showed increased expression of ICAM-1 and a light staining for ELAM-1 appeared on the superficial capillaries and venules of most patients. The expression of VCAM-1 increased slightly but did not change significantly.

Mesothelial cells. The mesothelial layer of patients with peritonitis was usually discontinuous with sheets of cells flaking away from the membrane. The remaining cells and the bare basement membrane were often covered with a membrane of hyaline material. Mesothelial cells from two patients were arranged in multiple layers interspersed with adherent leukocytes (Fig. 6). A few cells from these patients expressed the proliferation-associated antigen recognized by MoAb Ki-67. In contrast to the exclusive nuclear staining obtained in tonsil controls, Ki-67 produced nuclear and cytoplasmatic staining on mesothelial cells. As in the other groups, mesothelial cells expressed VCAM-1, ICAM-1, and TFR. Contrary to what one might expect, the expression of these molecules did not increase with peritonitis. Indeed, staining was significantly less for ICAM-1 and TFR.

Discussion

To date, studies of the mechanisms of peritoneal defense have concentrated on cells obtained from the dialysate effluent, and more recently on cultured mesothelial cells *in vitro*. The CAPD technique by its very nature produces a constant depletion and/or dilution of peritoneal fluid opsonins and phagocytes [5, 7, 11, 13]. As a consequence, their concentration in the CAPD fluid *in vivo* may not be sufficient, even after reaching a more physiological pH and osmolality, to clear invading micro-organisms in the fluid phase of the dialysis solution [5, 12, 13]. Despite these facts, a rate of one episode of peritonitis every nine months, nowadays considered by almost all as unacceptably high, represents on average only one episode every 1000 exchanges. Clearly other mechanisms must operate to account for this rather infrequent failure of the depleted peritoneal defense.

The present study attempts, for the first time, to combine information about peritoneal morphology with *in situ* characterization of resident and infiltrating immunocompetent cells in the peritoneal membrane as well as their spatial relation to endothelial and mesothelial cells. The initial supposition was that cells within the membrane might also be involved in the defense against infection. Although we have restricted our scope by studying exclusively the parietal peritoneal membrane, the assumption is that this area is representative of the participation of immunocompetent cells in surrounding tissue in the protection of the peritoneal cavity.

We have determined that leukocytes do not have a random spatial distribution within the peritoneal membrane. Instead, they display selective accumulation immediately under the mesothelial basement membrane and around the peritoneal blood vessels. As will be discussed below, cells from these two areas have distinct characteristics that suggest participation in different activities.

One of the most interesting findings of this study was that macrophages underlying the peritoneal basement membrane are arranged in close proximity to each other, forming an almost

continuous "first line" of cells with a phagocytic phenotype. The CD14 differentiation antigen used in this study is probably the best marker for cells of the mononuclear-phagocyte system, being detected on blood monocytes and tissue macrophages with great specificity [17]. However, CD14 is variably expressed in some types of dendritic cells involved in antigen presentation, that also express ICAM-1 and class II antigens [17, 18]. The 77 Kd antigen recognized by MoAb RFD7, on the other hand, is not expressed on dendritic cells [19] but shows a strong association with the phagocytic capability of macrophages [19, 20]. Likewise, CR1 is useful to discriminate between these cell types, since its is also not expressed on dendritic cells [21]. These submesothelial cells were therefore identified as macrophages on the basis of their expression of CD14, HLA-DR, TFR, ICAM-1, CR1, and the antigen recognized by MoAb RFD7.

We [20] and others [13] have detected large numbers of T lymphocytes in the peritoneal fluid. These cells must originate from the vessels that surround the peritoneal membrane. To gain access to the peritoneal cavity they must also transit across the endothelial layer, the perivascular and submesothelial areas and finally the mesothelial layer. In the omentum discrete lymphoid structures, called milky spots, are found early in life and during peritoneal infections [22]. Although they may contribute to the intraperitoneal T cell population, these foci of immune cells are not usually seen in normal adults. Despite possible differences between omental and parietal peritoneum, T cells were seldom seen in the submesothelial interstitium even during episodes of peritoneal infection.

On the other hand, T cells were regularly identified in the perivascular interstitium at the same areas where perivascular CD14-expressing cells also accumulated. Such proximity indicates a potential for interaction in processes such as antigen presentation/recognition or cell activation/maturation. In fact, T cell proliferation upon contact with macrophages has been previously documented within the omental peritoneal tissue after intraperitoneal administration of antigen [23]. In keeping with this hypothesis the perivascular area is the site of an intense inflammatory reaction during peritonitis and was also encountered in one patient on CAPD with a recent episode of peritonitis. A similar arrangement of perivascular macrophages and T cells, the dermal microvascular unit, which is possibly involved in antigen recognition, has been recently described in the normal human skin [24–26].

Most of the HLA-DR-expressing perivascular cells appear to belong to the mononuclear-phagocyte system on the basis of their expression of CD14. These cells also stained with MoAb RFD7, but the population seemed somewhat heterogeneous as a sizeable proportion of the cells did not express this antigen. This was further accentuated in patients on CAPD with or without peritonitis. Since we did not employ double staining methods and the semi-quantitative index used was not suitable for detecting differences in the proportional expression of different antigens in the same cell population, this fact became apparent only after the examination of consecutive sections stained for these antibodies.

The phenotype of CD14-positive and RFD7-antigen-negative macrophages is compatible with two hypotheses. The first is that these cells may be involved in antigen presentation to perivascular T cells rather than phagocytic activities. Another possibility is that they may represent an immature undifferentiated monocyte population that has recently crossed the endothelial lining. Such an explanation is consistent with their increased numbers in the dialyzed peritoneum: a constant influx of blood monocytes is expected to occur to compensate the continuous depletion of macrophages through the dialysate. This is also in agreement with previous reports showing that macrophages present in the effluent of CAPD patients may show signs of immaturity when compared to peritoneal macrophages from normal controls [6, 20].

Both in the control groups and in the CAPD patients, a greater proportion of macrophages from the submesothelial area displayed the RFD7 antigen when compared to those of the perivascular region. While we do not have proof, it is plausible to suppose that they can differentiate and become activated in contact with perivascular T cells before migrating to become incorporated into the macrophage pool underneath the mesothelium, or even further into the peritoneal cavity.

It may appear conceptually confusing that macrophages from CAPD patients can experience some degree of maturation as they leave the perivascular region and arrive at the submesothelial area, while at the same time, immature cells are found in the dialysis effluent [6, 20]. In a previous study [20], we have shown that the percentage of RFD7-expressing cells increased significantly when peritoneal macrophages from CAPD patients were compared with blood monocytes (33.5% vs. 5.3%), although they never reached the expression (more than 90% of the cells) found on peritoneal macrophages from normal individuals. It is not that mature macrophages disappear from the peritoneal cavity, just the population becomes more heterogeneous with an increased proportion of immature cells.

The results of the present study are to some extent in contrast with studies dealing exclusively with the morphology of the peritoneal membrane. The presence of macrophages or lymphocytes in the submesothelial tissue is rarely if ever mentioned either in animal or human peritoneum [27–31]. This discrepancy is likely to result from methodological differences. It has been said that macrophages are difficult to identify in tissue. They are usually scattered, can elongate or flatten and many times do not possess readily identifiable characteristics [17]. This description fits exactly with that of the peritoneal membrane macrophage. Indeed, in our biopsy material the number of times when "empty cytoplasmic extensions" with macrophage phenotypes were detected far outweighed those where a clearly identifiable nucleus could be seen amidst the stained cytoplasm. Due to their size and morphology the peritoneal macrophage covers a much wider area than the location of the nucleus would suggest. While this fact is probably relevant to their function, in addition it must contribute to underestimate their frequency within the peritoneal membrane. On the other hand, we have no explanation as to why the T-lymphocytes so often detected in the perivascular region have not been mentioned in previous morphological studies.

Again by analogy with the dermal microvascular unit [25, 26], endothelial cells from the normal parietal peritoneum express HLA-DR while resting endothelial cells are usually negative for this antigen [32]. Class II expression is induced almost exclusively by IFN-γ, a T cell product. HLA-DR expression on endothelial cells was clearly up-regulated during CAPD, particularly in patients with peritonitis. This provides indirect evidence for the existence of activated T cells secreting IFN-γ in the peritoneal membrane. Yet it is not clear whether class II up-regulation on peritoneal endothelial cells has a role in the peritoneal immune response, or is merely an epiphenomenon of the local release of

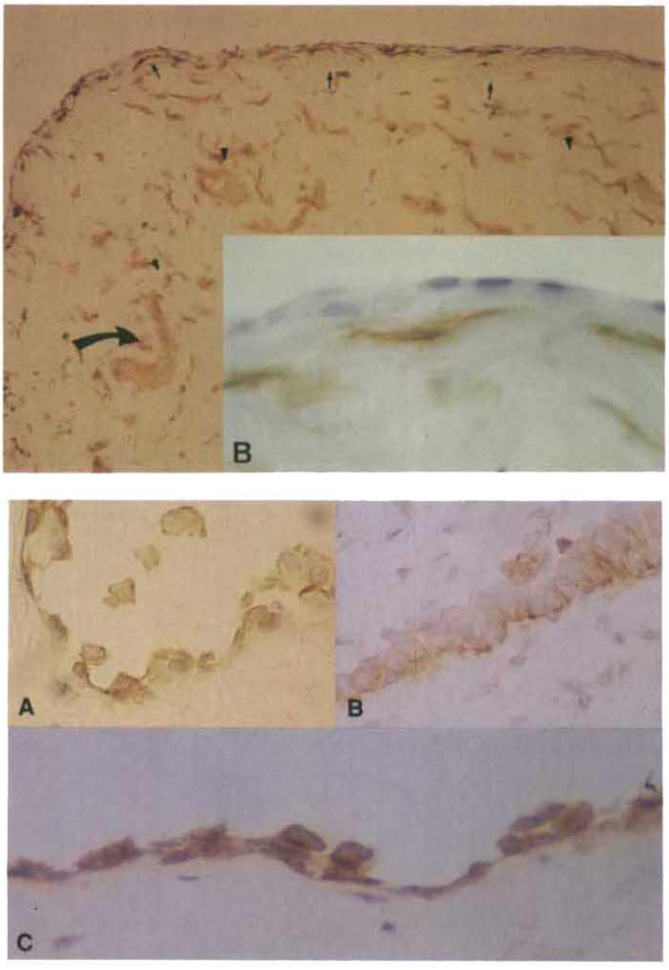

Fig. 3. A. *Mesothelial cells expressing ICAM-1 (normal control).* **B.** Mesothelial cells expressing VCAM-1. The peritoneal membrane is folded over itself so that two layers of mesothelial cells face each other (normal control). **C.** Mesothelial cells expressing the transferrin receptor (uremic control). Reproduction of this figure in color was made possible by a grant from Baxter Healthcare Corporation, Round Lake, IL.

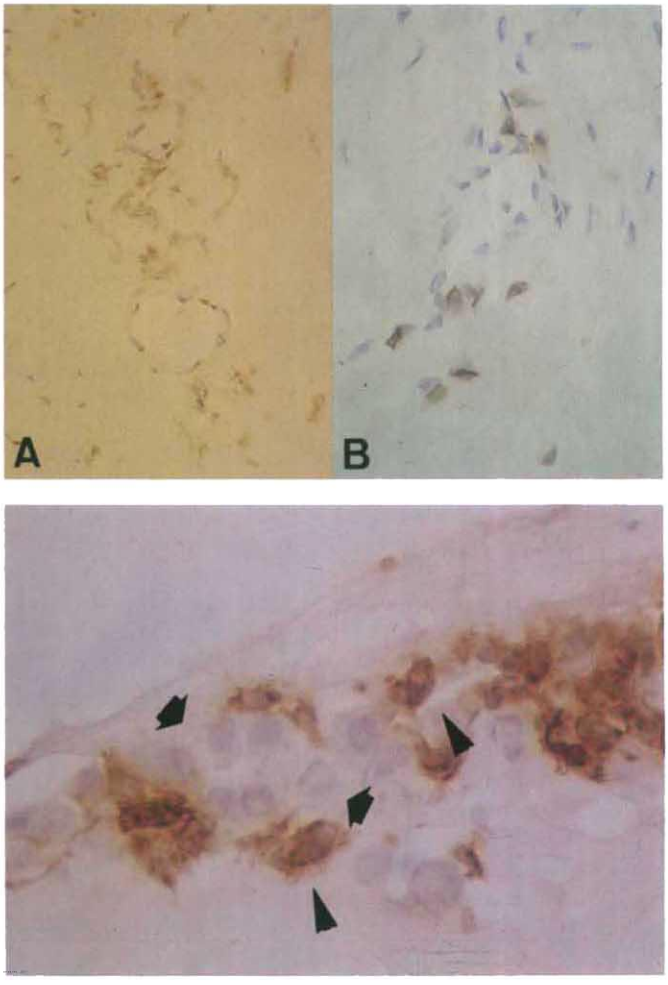

Fig. 6. *Patient with peritonitis.* Adherent HLA-DR expressing cells (arrowhead), identified on sequential sections as macrophages. Cells appear to accumulate above and below mesothelial cells arranged in multiple layers (arrow). Intracavitary macrophages adhere to themselves as well as to mesothelial cells. A loose amorphous material (presumably fibrin) overlays the peritoneal membrane. Reproduction of this figure in color was made possible by a grant from Baxter Healthcare Corporation, Round Lake, IL.

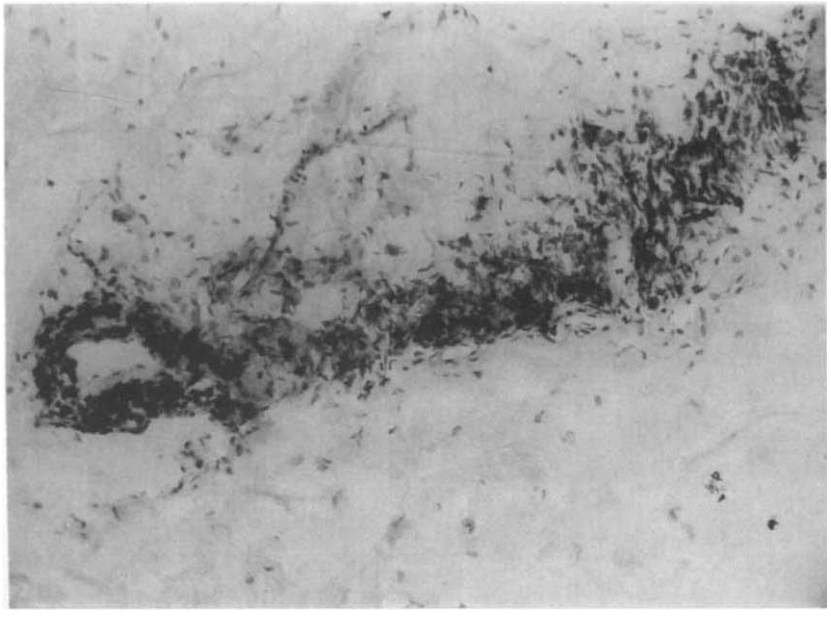

Fig. 4. *Dense vascular and perivascular T cell infiltrate in a patient with end-stage renal failure due to polycystic kidney disease.* The patient was on CAPD for seven months and had had only one episode of clinical peritonitis three months before the collection of the peritoneal biopsy (CD3 staining; UCHT1 antibody).

Fig. 1. A. *Peritoneal membrane from a normal individual stained for HLA-DR.* Positive cells are identified by the precipitation of the brown end product. An almost continuous line of positive submesothelial cells is seen underneath the mesothelium (arrow). Endothelial cells from the peritoneal blood vessels also reacts positively (curved arrows). Non-endothelial HLA-DR-positive cells (arrowheads) are seen arranged in a concentric fashion around blood vessels. **B.** Sequential section from the same area. Submesothelial cells with abundant cytoplasm and flattened appearance are identified as macrophages on the basis of CD14 (FMC32 antibody) staining. Reproduction of this figure in color was made possible by a grant from Baxter Healthcare Corporation, Round Lake, IL.
← Top, left

Fig. 2. A. *Perivascular cells belonging to the mononuclear phagocyte system expressing the CD14 (LeuM3 antibody) antigen in the normal peritoneal membrane (uremic control).* **B.** Perivascular T cells (stained with a mixture of CD2, CD7, CD8 and CD27 antibodies) in the normal peritoneal membrane (uremic control). Reproduction of this figure in color was made possible by a grant from Baxter Healthcare Corporation, Round Lake, IL.
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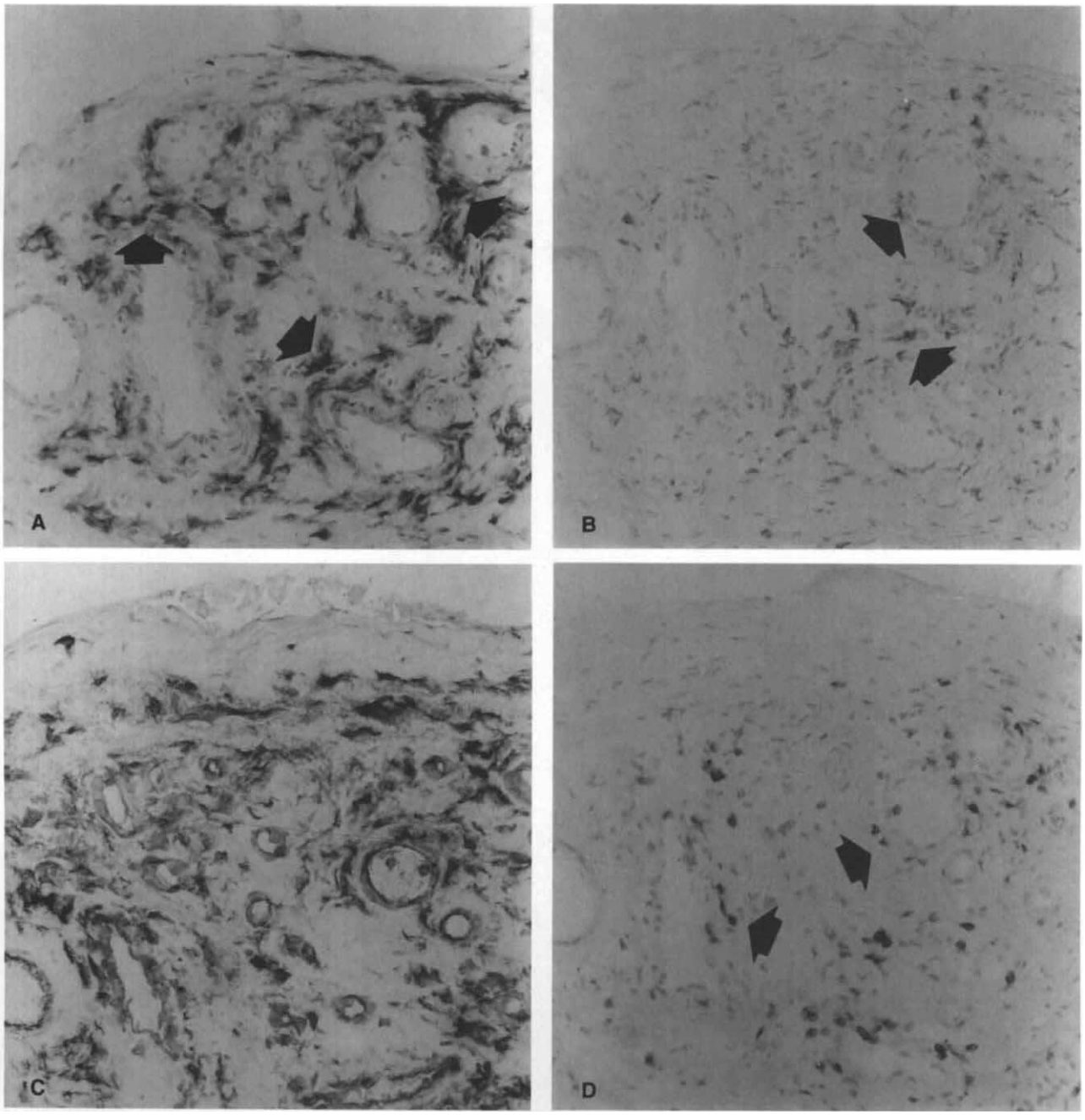

Fig. 5. *Serial peritoneal sections showing the perivascular infiltrate from a patient with peritonitis.* The infiltrate, identified in these black and white microphotographies by the dark end-product, is composed mainly of CD14-positive and RFD7 antigen-negative cells. **A.** CD14 antigen-positive mononuclear phagocytes stained with the FMC32 antibody (arrows). **B.** Few cells are stained for the RFD7 antigen (arrows). This suggests that most of the infiltrate is composed of recently emigrated monocytes. **C.** Strong HLA-DR up-regulation on both perivascular macrophages and endothelial cells. **D.** T cells (stained with a mixture of CD2, CD7, CD8 and CD27 antibodies) are found amidst the perivascular infiltrate (arrows).

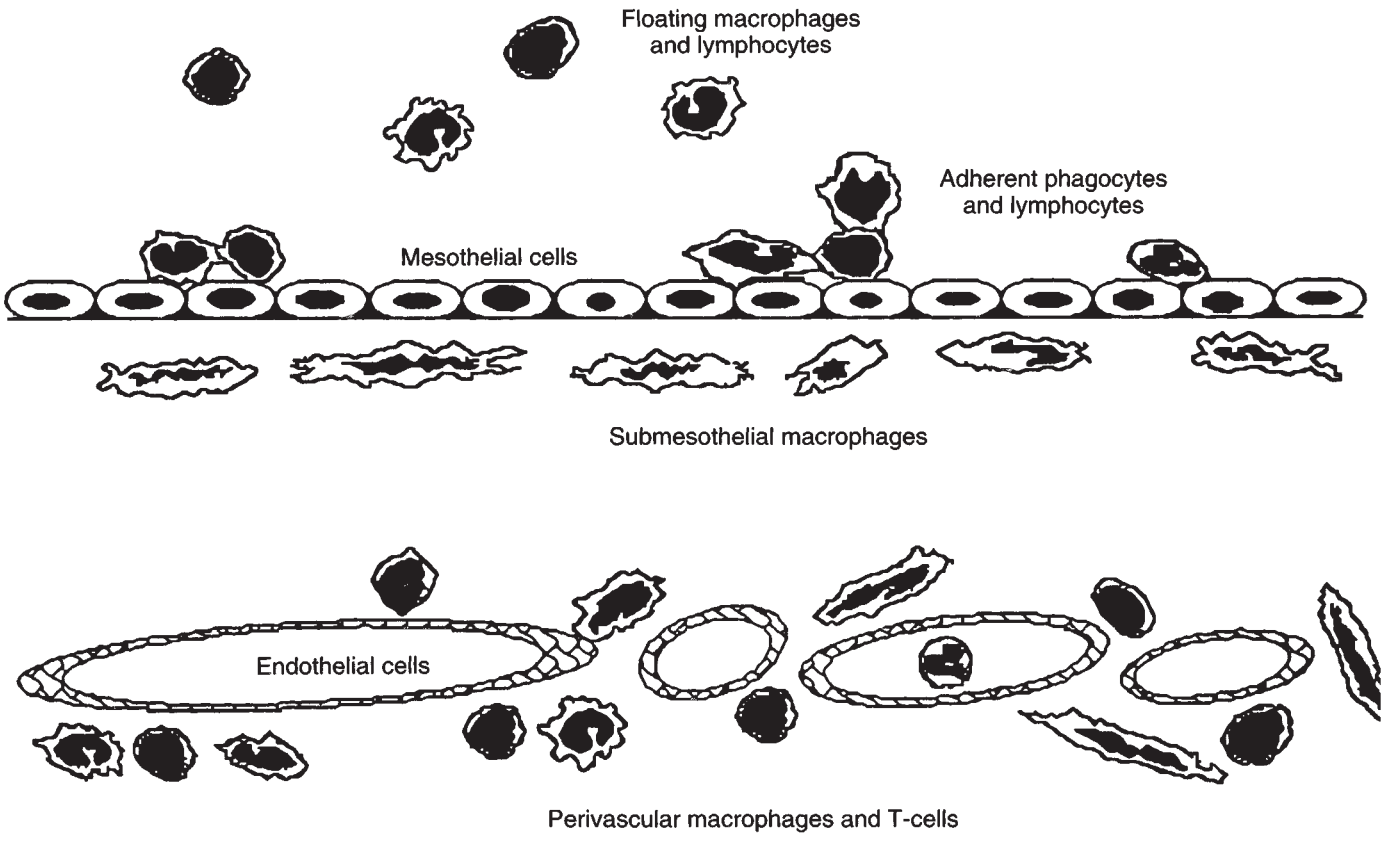

Fig. 7. *Proposed spatial distribution of immune cells in the peritoneal membrane and within the peritoneal cavity.*

cytokines. The characterization of a putative peritoneal microvascular unit involved in antigen recognition and cell-mediated immune response clearly merits further investigation.

The normal peritoneal endothelium also display moderate expression of the adhesion molecules ICAM-1 and VCAM-1. These cell surface molecules are involved in the adhesion of phagocytes and lymphocytes to endothelial surfaces and mediate cell extravasation during the inflammatory response where their expression is induced by a number of cytokines [33]. Their presence on the peritoneal microvasculature may at least in part account for the replenishment of immunocompetent cells continuously lost in the peritoneal dialysate. The up-regulation of these molecules in cases of peritonitis is consistent with this hypothesis.

Another adhesion molecule, ELAM-1—which mediates the binding of granulocytes to acutely inflamed endothelium [33]—was not present on endothelial cells from normal peritoneal membrane and only became lightly expressed in those cases with perivascular inflammation. The fact that our biopsies were taken at a relatively late phase of the infection may account for this fact as well as the rarity of neutrophils in patients with peritonitis. Accordingly, neutrophils within the peritoneal membrane were only identified in two of the cases showing ELAM-1 expression on endothelial cells. A recent report documents the participation of ELAM-1 in the binding of resting memory T cells to IL-1 induced endothelium [34]. The light expression detected in our patients may not have any relevance regarding cell-mediated responses, but it is tempting to speculate a role for ELAM-1 in the recruitment of memory T cells during peritoneal inflammation.

Normal mesothelial cells showed intense staining for the transferrin receptor, a finding that to our knowledge has not been described previously. Typically the TFR is expressed by cells in the process of multiplication [35]. However, the MoAb Ki-67 that recognizes an antigen present on the nuclei of proliferating cells [36] did not stain a single cell in the normal mesothelium. There was no evidence of a proliferative response by mesothelial cells, in the resting peritoneum, to explain their transferrin receptor expression. Ki-67 staining was found on mesothelial cells of only two patients with peritonitis. In these cases staining was localized to areas where mesothelial cells were arranged in multiple layers, displayed double nuclei and were, therefore, obviously undergoing active regeneration. Cells from these areas also strongly expressed the TFR. Hence, the intense TFR expression on mesothelial cells may have some other physiological role, and it is interesting that, apart from those patients with an actively regenerating mesothelium, TFR expression diminishes in most patients with peritonitis.

In contrast to cultured mesothelial cells [37], no HLA-DR expression was found on our biopsy material, even in those cases with ongoing peritonitis and strong up-regulation of this class II antigen on both endothelial cells and peritoneal macrophages (Fig. 6). However, we were able to detect the *in vivo* constitutive expression of the adhesion molecules ICAM-1 and VCAM-1 on mesothelial cells. Although the expression of these adhesion molecules has been reported on mesothelial cells in culture [37], the classical description of the tissue distribution of ICAM-1 does not mention this finding [38]. This is somewhat surprising as

samples of liver and intestine were studied and these organs have a mesothelial lining on the external surface. In contrast, a recent publication studying VCAM-1 does mention its expression on mesothelial cells of the liver peritoneum [39].

ICAM-1 is a counter-receptor for the β_2 integrin LFA-1 (CD11a/CD18) expressed on all leukocytes [40]. The interaction of ICAM-1 and LFA-1 provides accessory support to many lymphocyte and phagocyte interactions including antigen recognition and endothelial cell binding [33, 40]. Similarly, VCAM-1 through binding to the β_1 integrin VLA-4 also functions as an accessory molecule for lymphocyte and monocyte interactions with endothelial cells [33, 40].

It is reasonable to suppose that ICAM-1-LFA-1 and VCAM-1-VLA-4, and other as yet not identified pairs of surface molecules, are able to mediate lymphocyte and phagocyte binding to the mesothelial surface. In this manner, interactions between peritoneal cells would be able to proceed independently of the random collision of floating cells in the peritoneal fluid. Indeed, the encounter of phagocytes and bacteria upon tissue surfaces may be associated with more efficient phagocytosis, a fact previously shown *in vivo* on endothelial surfaces [41] and *in vitro* on cultured mesothelial cells [42].

More than 20 years ago, a microcinematographic study in mice described two different types of macrophages in the omental peritoneum: a submesothelial tissue bound dendritic cell, and a free, round or stellate cell that wanders on the surface of the peritoneal membrane [23]. In addition, leukocytes bound to the normal mice mesothelium have been demonstrated in a study where *in situ* fixation of the peritoneal membrane was employed [43]. More recently, monocytes have been shown to adhere to cultured human mesothelial cells, particularly after cytokine stimulation of either cell type, in a process where the adhesion molecules ICAM-1 and VCAM-1 and their ligands participate [37].

In spite of the suggestive evidence, the existence of leukocytes adherent to the normal human peritoneal membrane is still speculative. Adhesion may be weak under normal circumstances and, as such, unable to resist the handling required for harvesting and processing the peritoneal biopsy. Indeed, mesothelial cells, even with the support provided by their basement membrane, are particularly susceptible to detachment, unless the biopsy is collected with extreme care [14]. On the other hand, adherent cells are much easier to demonstrate during experimental [43, 44] and clinical peritonitis (Fig. 6) [44]. During acute peritonitis, not only leukocyte numbers are increased, but also up-regulation or activation of adhesion molecules on both cell types is likely to occur and prevent their detachment from one another.

In conclusion, in addition to mononuclear cells dispersed in the peritoneal fluid, we have shown the existence of two distinct peritoneal membrane leukocyte populations: the submesothelial macrophage and the perivascular unit composed of macrophages and T cells. We further show the constitutive expression of adhesion molecules on mesothelial cells *in vivo*, which suggests that intracavitary adherent leukocytes may also participate in the defense of the peritoneum. These four groups of immune cells and their histological localization are depicted in Figure 7. The investigation of the role of these different cells types should help to elucidate the mystery still posed by the peritoneal defense against invading microorganisms.

Acknowledgments

This work was partially supported by grant 406469/89-0 from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Dr. Suassuna was the recipient of an International Society of Nephrology Fellowship Training Award while in the UK and a scholarship from Coordenadoria de Aperfeiçoamento em Ensino Superior (CAPES) upon return to Brazil.

Preliminary parts of this work were presented at the 5th Congress of the International Society for Peritoneal Dialysis, Kyoto, July 1990 (published in OTA K et al: *Current Concepts in Peritoneal Dialysis. Excerpta Medica Congress Series*. 947:11–6 and 283–291, 1992) and at the 27th Congress of the European Dialysis and Transplant Association, Vienna, September 1990 (published in *Nephrol Dial Transplant* 5:721–722, 1990).

We thank Drs. Pedro P.X. Elsas, Elsa F. de Oliveira and José C. Sampaio, for their support in the conclusion of this project. We are also grateful to Ms. Janine Salter for technical assistance, to Mr. Renan M.V.R. Almeida for advice regarding the statistical analysis of the data, and to the many surgeons who assisted in the collection of the peritoneal biopsies.

The authors are grateful to Baxter Healthcare Corporation, Round Lake, Illinois, USA which sponsored publication of the color figures.

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