Urol Res (1992) 20:219–228

Urological Research

Morphological aspects of the interaction of Bacillus Calmette-Guérin with urothelial bladder cells in vivo and in vitro: relevance for antitumor activitiy?

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Accepted: November 1, 1991

Summary. Intravesical administration of Bacillus Calmette-Guérin (BCG) has been shown to be effective in the treatment of patients with superficial bladder cancer. For a better understanding of the mechanism of this antitumor activity, scanning and transmission electron microscope (SEM, TEM) studies were carried out to investigate morphological aspects of the interaction of BCG with the bladder wall in vivo and in vitro. Adherence of BCG to the bladder wall in vivo was studied 1 and 24 h after single or multiple (6×) BCG instillations in intact and in electrocauterized guinea pig bladders. Despite extensive search with SEM for its presence, virtually no BCG was found on the intact urothelium, and BCG was only occasionally observed in the coagulation lesions. SEM and TEM studies revealed adherence and phagocytosis of BCG by the T24 human bladder carcinoma cell line in vitro. Time sequence studies on the phagocytosis and fate of BCG showed that T24 cells are capable of progressively degrading the mycobacteria in phagolysosomes. However, BCG did not alter MHC class II antigen expression on T24 cells in vitro. In contrast, 54 urine sediments and bladder washings of 11 bladder cancer patients, taken prior to or after several intravesical BCG instillations, failed to demonstrate urothelial (tumor) cells showing evidence of BCG phagocytosis (682 cells screened by TEM), while BCG was phagocytized avidly by leukocytes. These data suggest that a direct interaction of BCG with urothelial bladder cells in vivo can be called in question.

Key words: Intravesical BCG – Bladder urothelium – Morphological interaction – Man – Guinea pig

Intravesical therapy of superficial bladder cancer in man with Bacillus Calmette-Guérin (BCG) was first reported by Morales et al. [17] and has since been proven to be an effective antitumor treatment for both recurrences of papillary tumors after endoscopic resection and for carcinoma in situ (see [21, 34] for reviews).

The immunostimulating and antitumor activities of BCG are well established [6, 8, 14]. It is generally assumed that BCG operates by an activation of the immune system. However, the actual mechanism of the antitumor activity of BCG in superficial bladder cancer has also still not been elucidated, and various effector mechanisms seem possible [15, 25, 34].

Repeated intravesical administration of BCG generates a granulomatous reaction in the bladder wall, as revealed by histological studies [11, 13, 28, 35]. Immunohistochemical and flow-cytofluorometric studies have shown that T-lymphocytes, monocytes/macrophages and polymorphonuclear leukocytes are the main infiltrating cells in response to intravesical BCG treatment [7, 13, 24]. Furthermore, BCG treatment induces a class II MHC (HLA-DR) antigen expression on urothelial tumor cells, thus altering the phenotype of the tumor cells, with possible implications for the mechanism of antitumor activity of BCG [2, 9, 23, 33].

In several animal tumor models the intratumoral administration, i.e., local presence of BCG, has been shown to be effective [8], suggesting that a close contact of BCG with tumor cells is important. However, little is known about the interaction between BCG and the bladder wall. It is conceivable that the initiation of interaction takes place by way of attachment to the bladder mucosa, possibly mediated by a bacterial exopolysaccharide glycocalyx [16], and it has been suggested that host fibronectin may play a role in the adherence of BCG to the bladder wall [26, 27]. In addition, very recently the internalization of BCG by bladder tumor cells has been described [1].

In the studies reported here we investigated morphological aspects of the possible interaction of BCG with normal urothelium and bladder tumor cells using light microscopy and scanning and transmission electron microscopy (SEM, TEM). We studied the adhering capacity of BCG to guinea pig bladder in vivo, and the phagocytizing capacity of human bladder tumor cells and the fate of BCG, both in vivo in BCG-treated patients and in vitro.

Materials and methods

Animals

Female Sewall Wright inbred strain 2 guinea pigs, 3–4 months old and weighing 300–400 g were used for the experiments. The animals were originally obtained from the NCI-Frederick Cancer Research Facility, Frederick (Md.), USA, and were bred at the Laboratory Animal Facility of the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. The animals were fed with normal guinea pig ration (Hope Farms, Woerden, The Netherlands) and water ad libitum.

Bladder tumor cell line

The T24 bladder carcinoma cell line [4] was a gift from the Dept. of Urology, St. Radboud Hospital, Catholic University of Nijmegen, The Netherlands (Prof. Dr. F. M. J. Debruyne). T24 cells were propagated in RPMI-1640 medium containing 25 mM Hepes buffer (Gibco Europe, Breda, The Netherlands), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 0.5% 0.2 M L-glutamine, 100 IU/ml penicillin (Gist Brocades, Delft, The Netherlands) and 100 μg/ml streptomycin (Sigma Chemie Benelux, Axel, The Netherlands), hereafter referred to as complete RPMI. Cells were propagated at 37°C in a 5% CO_2 atmosphere. For passage, the cells were trypsinized with 0.25% trypsin plus 0.2% EDTA in PBS.

Bacillus Calmette-Guérin

Several batches of BCG preparations produced by the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands, were used for the experiments. BCG-RIVM had been produced in a homogeneously dispersed culture and was harvested at the end of logarithmic growth; the original seedlot had been obtained from the Institute Pasteur, Paris, France.

Immediately before use in in vitro experiments, BCG was resuspended in sterile aqua dest., thoroughly washed repeatedly by centrifugation to remove Haemaccel and Tween 80, finally resuspended in PBS or complete RPMI (without antibiotics added), and dispersed ultrasonically to reduce the lumps representing the culturable particles. In some experiments a BCG-Tice (Health Science Center, Chicago, Ill.) preparation was used which had been produced as a surface pellicle.

Urine sediments and bladder washings from patients

Bladder cells were obtained from bladder cancer patients receiving six instillations of BCG at 1-week intervals. Urine sediment cells were collected from spontaneously voided urine of five patients with carcinoma in situ (CIS) of the bladder urothelium. Urine specimens were obtained before and at several time-points from 1 to 24 h after instillations with 5×10^8 cfu BCG. Bladder lavage cells were collected by bladder washing from seven patients with CIS or high-grade (GII/GIII) superficially papillary bladder cancer. The bladder washings were performed before and occasionally also after BCG instillations by irrigation of the bladder with 50 ml saline twice.

Urine and bladder washing specimens were kept cool (4°C or on melting ice) until preparation. For studies, the cells were centrifuged (10 min, 300 *g*) and washed twice and resuspended in PBS. Some of the cells were used for preparation of cytospins, and the others were processed for TEM.

Adherence of BCG to urothelium in vivo – experimental design

Guinea pigs were used to examine the possible adherence of BCG to normal or superficially damaged bladder urothelium. Briefly, to obtain a damaged coagulated bladder surface (mimicking transurethral resection wounds and possibly also carcinoma in situ in man) electrocautery was performed, followed immediately by intravesical instillation of BCG. After 1 h the BCG was removed. Bladder tissue for SEM studies was collected immediately or 24 h after the BCG removal.

Electrocautery. Animals were anesthesized by an i.m. injection in the hindleg of 0.6–0.8 ml of a 7:3:1 mixture of Ketalar (150 μg/ml; Parke Davis, Madrid, Spain), Rompun (2.5 mg/ml; Bayer, Leverkusen, FRG) and atropine (0.05 mg/ml). The back was shaved and wet with tap water (for electrical conductance). The bladder was emptied by careful manual pression. The animal was positioned dorsally on a moistened metal plate and the vulva was disinfected with 0.02% hibitane. Bladders were catheterized using a Teflon sheath of an Abbocath-T20 gauge infusion needle (Abbott Ireland, Sligo, Ireland), provided with a flexible metal wire (Cordis, Miami, Fla) or with a curved, blunt needle (Abbott, 20 gauge) using Instillagel (Farco-Pharma, Cologne, FRG). The wire or needle was pressed against the bladder wall, and with an electrode of a coagulation apparatus (Electrotom 120, Martin Elektromedizin, Tuttlingen, FRG) electrical contact was made (wire: 4 s at position 2; needle: 0.2 s at position 3), causing a burn in the bladder wall. After this procedure the catheter was removed.

BCG instillation. Intravesical administration of BCG was done as previously described [35] and under the same anesthesia. Bladders were catheterized with Teflon sheaths of Abbocath-T24 gauge infusion needles and 5×10^7 cfu of BCG resuspended in 1 ml sterile PBS was instilled through this catheter.

After the BCG had been in the bladder for 1 h and with the animal still under anesthesia, the bladder was carefully emptied by gentle compression of the suprapubic region. Control animals were treated similarly, using sterile PBS for the instillation. Autopsy was performed immediately after the removal of BCG or 24 h later. In one experiment the BCG instillation was repeated at weekly intervals up to a total of six instillations, and autopsy was done 24 h after the last instillation. Details of the experimental design used in subsequent studies are given in Table 1.

Autopsy. At autopsy, the complete bladder was removed and fixed by instillation and immersion with fixative (stretched urothelium). Alternatively, the bladder was cut into two halves, taking care that the coagulation lesion was localized completely in one of the halves, and the halves were pinned flat on cork and fixed by immersion under fixative (folded urothelium). Subsequently, 5×5 mm tissue pieces were excised and selected, taking care that both normal and injured, and dorsal as well as ventral bladder wall parts were collected for scanning electron microscopy.

BCG-T24 bladder tumor cell interaction in vitro – experimental design

For BCG adherence and phagocytosis experiments the T24 bladder carcinoma cells were grown in 6- or 12-well plastic plates (Costar 3506, Cambridge, Mass.) in complete RPMI, without antibiotics added. The number of T24 cells seeded in the wells was dependent on the total duration of the in vitro culture, in such a way that when cells were collected for SEM and TEM studies a complete monolayer could be expected. For SEM, small round sterile cover glasses (Ø 15 mm) were placed on the bottom of the wells. The T24 cells were seeded at day −1 and were allowed to settle for 24h. After removal of the medium at day 0, BCG in fresh complete RPMI (without

Table 1. The presence of mycobacteria after intravesical BCG-RIVM instillation on intact or electrocauterized guinea-pig bladder urothelium, as estimated by scanning electron microscopy (SEM)

Expt. no.	Electro-cautery	Number of BCG instil-lations	Autopsy at	No. of animals in expt	Presence of mycobacteria per animal	
					On normal urothelium	In coagulation lesion
1	–	1	1 h	3	0/3	–
2	+[a]	1	1 h	4	0/4	1/4 (±)[c]
	+[a]	1	24 h	4	0/4	0/4
3	+[b]	6	24 h	6	0/6	–[d]
4	+[b]	1	1 h	4	1/4 (±)	1/4 (++)[c]
	+[b]	1	24 h	4	0/4	2/4 (±)[c]
						2/4?[e]

[a] Coagulation of flexible metal wire, see Materials and methods
[b] Coagulation by blunt, curved needle, see Materials and methods
[c] ±, ++ score, indicating the numbers of BCG found; ± few/++ moderate number
[d] Coagulation lesion healed (6 weeks after electrocautery)
[e] SEM preparations not suitable for examination

antibiotics) was inoculated onto the cells at a concentration of about 1.5×10^6 cfu/cm^2 (6-well plates: 1 ml of 3×10^7 cfu/ml per well; 12-well plates: 0.5 ml of 3×10^7 cfu/ml per well). The plates were centrifuged for 2 min at 600 *g* (Minifuge RF, Heraeus Christ, Osterode, FRG) to aid contact between BCG and T24 cells.

After 24 h (day 1) the cultures were washed four times with the complete RPMI without antibiotics to remove non-adherent, non-phagocytized BCG. Cells destined for the study of adherence and phagocytosis after 24 h were harvested for electron microscopy (EM), and the remaining wells with cells were filled with fresh culture medium without antibiotics. Further T24 cells were harvested at days 2, 3, 4, 5 and 6 after BCG inoculation. For SEM monolayers of T24 cells grown on cover glass were fixed in situ; for TEM cells in the wells were trypsinized, centrifuged for 7 min at 300 *g* (Biofuge A, Heraeus-Christ) and fixed as pellets. T24 cells inoculated without BCG were included for growth control purposes. Furthermore, culture media removed from the wells prior to the cell harvesting were tested for contaminant bacterial growth, and were found negative.

Cytospin preparation and staining

Cells in 100 µl PBS were cytocentrifuged on slides. The slides were air-dried, fixed for 5 min in methanol and subsequently stained according to the Ziehl-Neelsen method for prescreening of the samples on the presence of acid-fast bacteria. For this, the cells were stained 30 min with heated carbol-fuchsine, destained for 10 min with 1.3% HCl in ethanol 96% and counterstained with methylene blue. Intermediate washings were performed with tap water.

Scanning electron microscopy

A standard procedure for SEM preparation was followed. Briefly, the bladder tissue samples were fixed for 3–18 h and the T24 cell cover glass monolayers for 30–60 min at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After washing in several changes of the fixative buffer + 0.1 M sucrose, the samples were postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4), followed by 2% tannic acid (cat. no. 1764, Malinckrodt, Paris, K.) in the same buffer and again 2% OsO_4 for 1 h each at 4°C, with washing steps in between. The samples were dehydrated in a graded series of acetone or ethanol, ending with 100% for 20 min twice, and critical point dried using CO_2 in a Polaron E 3000 critical point drying apparatus, mounted on aluminum stubs, and gold-sputtered in a Polaron E 5000 sputter coater. The specimens were examined in a Philips 501 B SEM operated at 7.2–30 kV.

Transmission electron microscopy

Pellets of T24 cells and urine sediment or bladder washing pellets were fixed in 2.5% glutaraldehyde as above for 60–90 min at 4°C. Occasionally, the urine sediment or bladder washing pellets were very loose, making agar embedding after primary fixation necessary. The samples were postfixed in 2% OsO_4 + 1.5% $K_4Fe(CN)_6$ in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4°C, dehydrated in a graded series of ethanol and embedded in Spurr epoxy resin, medium hardness. Semithin (1 µm) sections for light microscopy were prepared and stained with toluidine blue, and thin sections for TEM were stained routinely with uranyl acetate followed by lead citrate. The specimens were examined in a Philips EM201 or 400 T TEM operated at 60 kV.

Determination of MHC class I and II antigens on T24 cells

T24 cells were cultured for 6 days in complete RPMI, with or without 5000 U/ml interferon gamma (IFN gamma; recombinant gamma interferon: Boehringer Ingelheim, Ingelheim am Rhein, FRG) and with or without BCG-RIVM (3×10^5 cfu or 3×10^7 cfu/well) in 6-well plates. After trypsinization and counting (0.5% trypan blue exclusion), 5×10^5 cells in 50 µl PBS with 0.01% NaN_3 and 2% FCS were labeled with 50 µl MoAb anti-class I-FITC (1:200 in PBS + NaN_3 + FCS, SeraLab, Crawley Down, England), MoAb anti-class II-FITC (1:5 in PBS + NaN_3 + FCS, Becton Dickinson, Etten-Leur, The Netherlands), or control MoAb (anti-KLH-FITC, 1:5, Becton Dickinson) in 96-well Microtest III Assay Plates (Becton Dickinson). Expression of class I and II antigens was determined by flow cytofluorometric analysis (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, Calif.).

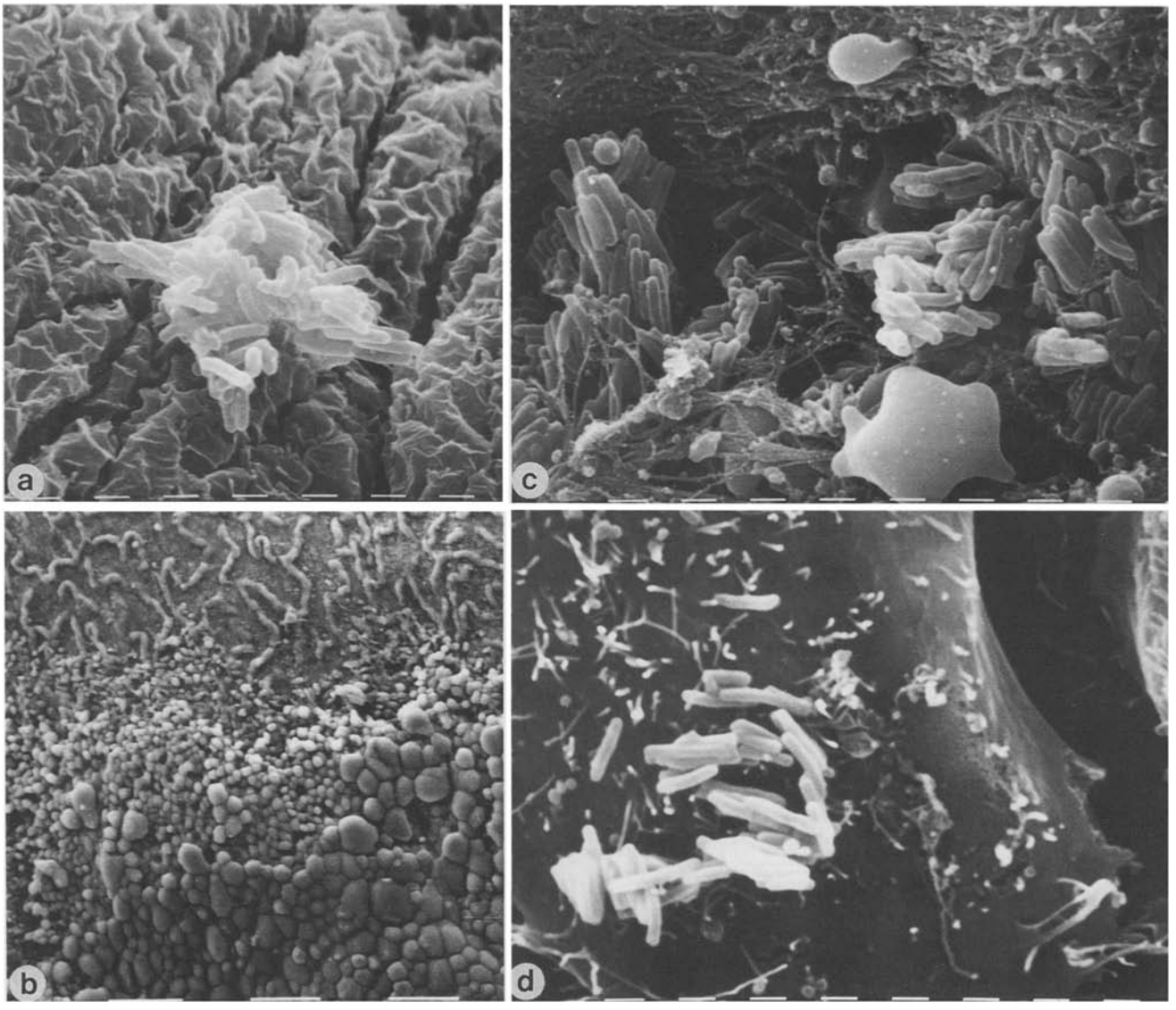

Fig. 1a–d. SEM of adherence of BCG in vivo and in vitro. **a** Shows BCG present on the surface of a normal superficial guinea-pig bladder urothelial cell. **b** Survey of the margin of a coagulation lesion caused by electrocautery, with swollen superficial urothelial cells at the bottom, a zone of smaller intermediate urothelial cells and inflammatory cells in the central part, and a bare basal lamina with protruding anastomosing capillaries at the top of the micrograph. **c** BCG presence on bare basal lamina in the margin of a coagulation lesion. **d** Adherence of BCG to the surface of a T24 human bladder carcinoma cell in vitro. Note the occurrence of BCG in characteristic lumps (**a, c, d**). *Bars,* 1 μm (**a, c, d**) or 100 μm (**b**)

Results

Adherence of BCG to guinea pig urothelium in vivo

SEM studies were carried out to investigate the possible sites and the nature of contact of intravesically instilled BCG with the intact or artificially damaged guinea pig inner bladder wall. Details of the experimental design and the results of these experiments are summarized in Table 1. The search for BCG concerned mainly BCG lumps, since these are very characteristic in shape, frequently present in the BCG preparation and far easier to find than single mycobacteria. No BCG was found on the surface of the normal guinea pig bladders after 1 h instillation (expt. 1). Similarly, virtually no BCG was observed on normal urothelium directly or 24 h after a single 1-h BCG instillation in bladders in which a coagulation lesion was inflicted by electrocautery (expts 2 and 4). In 1 bladder only among the 16 examined, a few isolated lumps of BCG were detected on the normal urothelial surface (Fig. 1a).

The coagulation lesions were characterized by the central absence of urothelium, leaving bare submucosal connective tissue with numerous profiles of small subepithelial capillaries, some inflammatory cells and red blood cells exposed, and a peripheral zone with gradually less damage to the transitional epithelium (Fig. 1b). In most of the bladders BCG could not be detected in the coagulation lesion either, but in 4 out of the 16 bladders

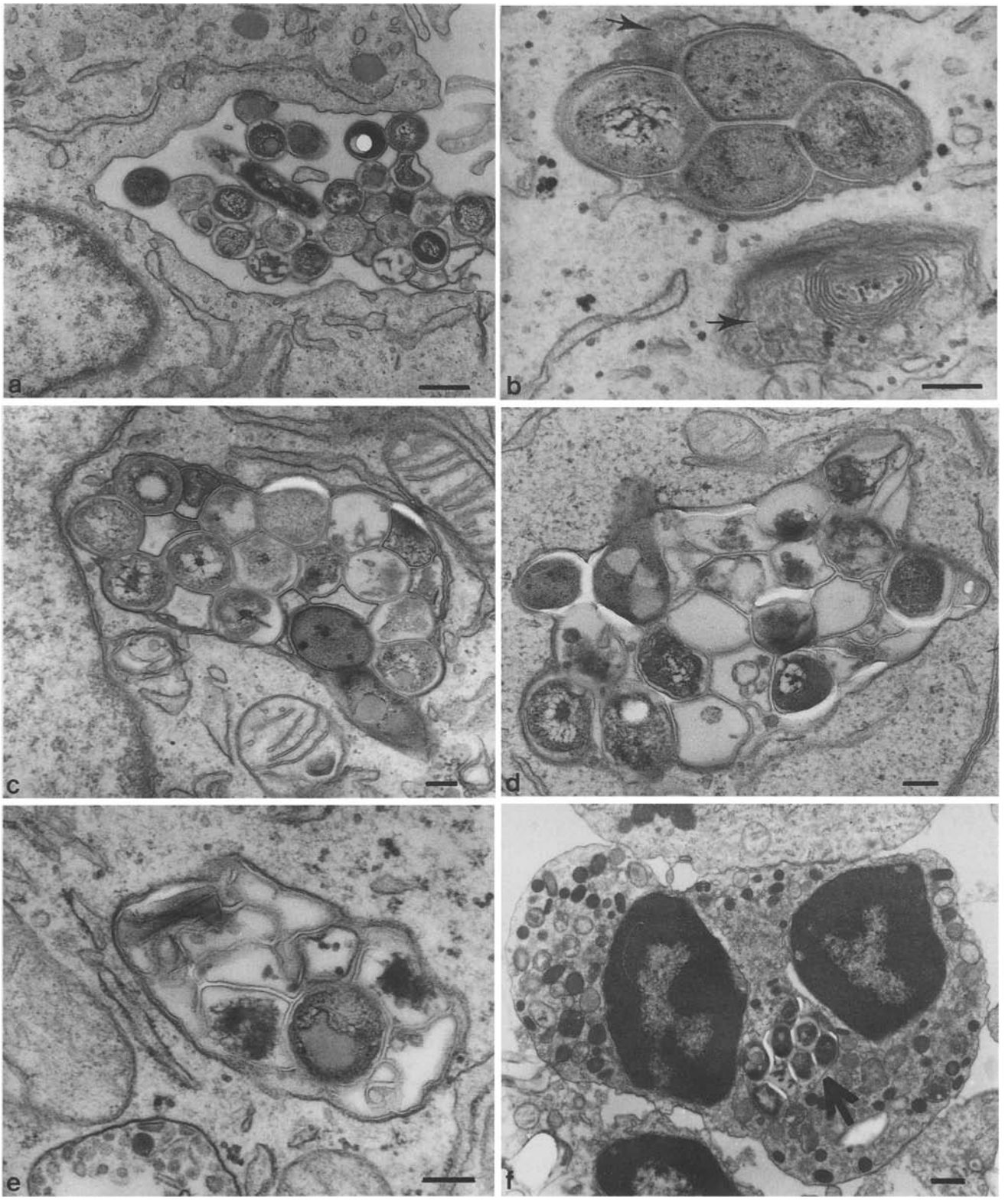

Fig. 2a–f. TEM of phagocytosis and intracellular digestion of BCG by (**a–e**) T24 human bladder carcinoma cells in vitro, and (**f**) neutrophilic PMN in vivo. **a** Start of phagocytosis of a lump of BCG by the T24 cell. **b** Phagocytized BCG in a membrane-bound vesicle which can be identified as a phagolysosome because of the presence of structures *(arrows)* observed also in a clear lysosome *(bottom right).* **c, d, e** Progressive degradation of BCG with granulation, vacuolation, coagulation and solubilization of the mycobacterial cytoplasm, leaving empty cell wall profiles as the last recognizable remnant of BCG. **f** Human neutrophilic polymorphonuclear leukocyte with phagocytized BCG *(arrow),* from a bladder washing of a patient 5 h after the second intravesical BCG instillation. *Bars,* 0.5 μm (**a, f**) or 0.2 μm (**b–e**)

Table 2. Ultrastructural quantification of adherence and phagocytosis of BCG by T24 bladder carcinoma cells in vitro[a]

BCG preparation	Adherence SEM	Phagocytosis TEM	
	Cells with adherent mycobacteria	T24 cells (%) with phagocytized BCG	Number of phagolysosomes per phagocytizing T24 cell
RIVM	67	64	2.6 ± 1.9[b]
TICE	67	71	3.0 ± 2.2

[a]T24 cells were incubated for 24 h with mycobacteria, and 100 cells were investigated with scanning (SEM) and transmission electron microscopy (TEM)
[b]Means ± sd

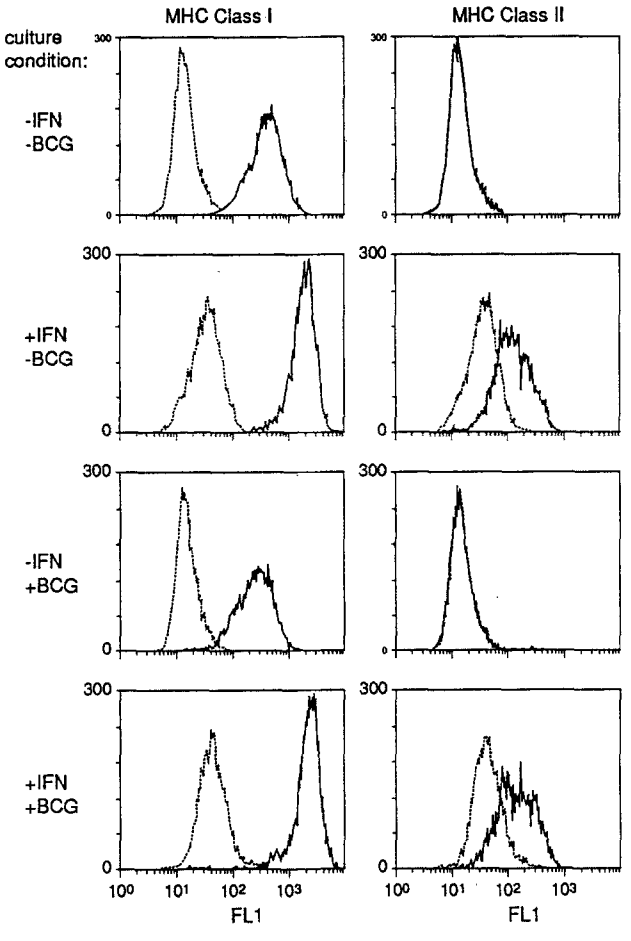

Fig. 3. Expression of MHC class I and II antigens on T24 bladder carcinoma cells after 6 days of culture with or without IFN gamma (5000 U/ml) and/or BCG (3×10^5 cfu/well). Fluorescence was measured by flow cytometry after labelling with anti-MHC class I or II MoAb (—) or control MoAb (···)

(expts 2 and 4) occasional BCG lumps were found at this site (Fig. 1c). In 1 bladder a moderate number of BCG cells and lumps was even found in the lesion. If present at all, the mycobacteria were mainly found at the margins of the coagulation lesion. No particular structural characteristics could be established which accompanied the presence of BCG at the deposits.

In the bladders subjected to six consecutive weekly BCG instillations (expt. 3), no BCG was found on the urothelial surface, as the coagulation lesions had healed in the meantime and were therefore not longer discernible.

Table 3. Summary of urine sediments and bladder washings collected from cancer patients undergoing intravesical treatment with ca. 5×10^8 BCG-RIVM for 1 h. Presence of BCG in these specimens as determined by cytology (cytospins, 1-μm epoxy sections) and TEM

Patient no.	Tumor	BCG instillation no./sampling time/ presence of BCG[a]	
		Urine sediments	Bladder washings
1	CIS	III/0[b], 1, 5, 24	
2	CIS	VIII/0, 1, 5, 24	
3	CIS	I/0, 1, 5, 24 II/0, 1, 5, 24	
4	CIS	II/0, 7, 24	
5	CIS	I/0, 5, 20 II/0, 5, 20	I/0, 5, 20 II/0, 5, 20
6	pap./TUR		III/0, IV/0, V/0
7	pap./TUR		V/0, VI/0
8	pap./TUR		VI/0
9	pap./TUR		V/0
10	pap./TUR		I/0, II/0, III/0, IV/0, V/0, VI/0
11	CIS		II/0, 4; III/0, 4, IV/0, 4; V/0, 4; VI/0, 4

[a]Roman numerals express the BCG instillation serial number; the numerical notations thereafter represent the time points (h) of sampling relative to the BCG instillation concerned. The samples in which BCG was found are underlined
[b]0 stands for pre-instillation samples, i.e. samples obtained just prior to the (next) BCG instillation

Adherence and phagocytosis of BCG by T24 bladder carcinoma cells in vitro

The adherence of BCG to T24 cells was studied by using SEM to score the number of T24 cells showing mycobacteria adhering to their surface. Two thirds of all T24 cells appeared to possess single or grouped adherent mycobacteria after 24 h incubation (Fig. 1d), regardless of the BCG origin (RIVM or Tice; see Table 2). Maximal adherence was observed at as little as 4 h (data not shown).

Quantification by TEM of the number of T24 cells showing phagocytized BCG revealed a quite comparable percentage of T24 cells that had phagocytized mycobacteria. Moreover, the number of phago(lyso)somes containing BCG per T24 cell after a 24-h incubation period as determined in ultrathin sections appeared to be comparable for BCGs from different sources (Table 2).

Table 4. (Semi-)quantitative cytologic evaluation of the presence of BCG in urine sediments or bladder washings from 11 bladder cancer patients undergoing intravesical treatment with ca. 5×10^8 BCG for 1 h[a]

Samples	Presence of BCG			
	Number of samples/total	Non-phagocytized[b]	Phagocytized[b]	
			PMN/macrophages	Urothelial (tumor) cells
Urine sediments (patients 1–5)				
Preinstillation	0/7	−	−	−
1 h postinstill.	4/4	++(+) (4)	+ (1)	−
5–7 h postinstill.	4/7	+ (4)	±/+ (4)	−
20–24 h postinstill.	1/7	−	± (1)[c]	−
Total	9/25			[424 cells]
Bladder washings (patients 5–11)				
Preinstillation	5/20	±/+ (3)	± (3)	−
4–5 h postinstill.	5/7	+ (+) (3)	± (4)[c]	−
20 h postinstill.	1/2	−	± (1)[c]	−
Total	11/29			[258 cells]

[a]Summation of observations on cytospins, 1-μm epoxy sections and TEM sections
[b]Score: − none, ± incidental, + few, ++(+) moderate to many BCG present. Numbers between brackets represent no. of samples showing feature
[c]Based on incidental observation of PMN-phagocytized BCG

In addition, the kinetics of the phagocytosis and eventual fate of BCG in T24 human bladder carcinoma cells were studied by TEM in a time-sequence experiment lasting until 5 days after 24 h of incubation with BCG. Mycobacteria adhering to T24 cells and/or involved in the process of phagocytosis (Fig. 2a) were noted occasionally throughout the experiment owing to the imperfections of the washing procedure to remove free, non-phagocytized mycobacteria after the 24-h incubation period. Ultrathin sections of the T24 cells at all stages from 0 to 5 days after 24 h BCG inoculation showed phagocytized single mycobacteria or groups of bacteria (Fig. 2b–e). The mycobacteria within the T24 cells were present without exception in membrane-bound vacuoles that could be identified either as phagosomes or, mainly, as phagolysosomes owing to the presence of vesicular and granular contents such as those also seen in plain lysosomal profiles (Fig. 2b).

Mycobacteria within the phagolysosomes were in various states of structural integrity, ranging from completely intact to largely injured, indicating the gradual degradation of BCG (Fig. 2c, d, e). Some morphological damage to mycobacteria was already apparent after 24 h of incubation of the T24 cells with BCG; relatively more mycobacteria in phagolysosomes were affected at later stages, although intact bacterial cells were also still noted after 6 days. The degradation of the mycobacteria was characterized morphologically by a progressive coarsening of cytoplasmic granulation, vesiculation, formation of electron-dense and electron-lucent cytoplasmic compartments (Fig. 2c, d), solubilization, and finally empty cell wall profiles that were sometimes hardly recognizable as BCG remnants (Fig. 2e).

MHC class I and II expression on T24 cells

In addition to EM studies, we investigated whether the ingestion and subsequent degradation of BCG by T24 bladder carcinoma cells influenced MHC expression. Figure 3 shows that MHC class I expression on T24 cells (Fig. 3a) is increased by IFN gamma (Fig. 3c). However, BCG did not alter the natural MHC class I expression (Fig. 3e) or that of IFN gamma-increased MHC class I (Fig. 3g). MHC class II antigens were not present on T24 cells (Fig. 3b), but induction was seen after culture with IFN gamma (Fig. 3d). No alteration of class II expression was observed to follow incubation with BCG (Fig. 3f, h).

Urine sediments and bladder washings from bladder cancer patients after BCG instillations

Cytologic investigation of cytospins and 1 μm sections of epoxy-embedded urine sediment specimens, obtained immediately before and at several time points after various BCG instillations, revealed the presence of BCG in 9 out of 18 (mainly early) postinstillation urine sediments only, while all 7 preinstillation specimens were negative (Tables 3, 4). TEM confirmed that BCG was either present as free culturable particles or was associated

with cells that could be mainly identified as polymorphonuclear leukocytes (PMN neutrophils) (Table 4). Despite careful search BCG was never found internalized by normal or malignant urothelial cells, which were observed in variable numbers.

As information became available (T. L. Ratliff, personal communication) that BCG could be found in bladder washings just prior to subsequent instillations, i.e. 7 days after the previous instillation, preinstillation bladder washings from 7 patients were studied subsequently (Table 3). BCG was found in 5 out of 20 of these washings. In addition, 6 out of 9 post-instillation bladder washings from 2 of the patients were also BCG-positive. Here too, loose BCG culturable particles and/or BCG internalized in PMN granulocytes were concerned (Table 4, Fig. 2f). Not once was BCG found in relation to exfoliated urothelial cells or cell lumps. A total of 682 urothelial (tumor) cells, from urine sediments and bladder washings together, were screened by TEM for the presence of phagocytized and degrading BCG, with negative result.

Discussion

The mechanism of the antitumor activity of BCG, although known to occur by way of stimulation of the immune system, has still not been elucidated [6, 8, 14]. One of its applications, intravesical BCG administration in patients with superficial bladder cancer, is also thought to be a form of non-specific immunotherapy, but its mechanism is far from understood [15, 25, 34]. The effectiveness of local administration of BCG suggests that a close contact or other direct interaction with the tumor cells or other (normal) cells of the bladder wall may be important for induction of the inflammatory and immune responses. However, little is known about morphological aspects of the possible interaction of BCG with the bladder wall, and this was the subject of the present study.

Using SEM and TEM, cellular interactions between BCG and normal and malignant bladder urothelium cells were studied in vivo and in vitro. The present report suggests that there is no convincing morphological evidence for a close interaction in vivo; adherence experiments in guinea pig bladders and urine sediments/bladder washings from bladder cancer patients treated intravesically with BCG failed to show a clear direct contact between BCG and intact urothelium or individual urothelial cells. In contrast, the human T24 bladder carcinoma cell line was shown to be capable of adhering, ingesting and degrading BCG in vitro.

The SEM findings with regard to the in vivo adherence of mycobacteria to guinea pig bladder walls 1 or 24 h after single or multiple BCG instillations were disappointing, especially as the search extended over at least 10–25% of the total ventral and dorsal bladder surfaces. The discovery of so few mycobacteria, particularly immediately after a 1-h instillation period with 5×10^7 cfu BCG, makes the necessity of an adherence process as the starting point for a cascade of events leading to antitumor activity questionable. Taking into account that a clear inflammatory reaction can be induced in the bladder wall by repeated instillations of BCG in normal intact bladders [11, 13, 28, 35], the possibility cannot be excluded that an interaction with some product released by BCG is the triggering factor for this process, rather than BCG adherence to the urothelium.

Apart from this, the virtual absence of mycobacteria from the intact bladder surface and the sparse presence at electrocauterized bladder sites appear to be in agreement with observations by Ratliff et al. [26] that adherence is minimal in normal mouse bladders and is increased 100-fold or more in bladders injured by electrocautery. It has been suggested that fibronectin is involved in the attachment of BCG to the bladder wall [26, 27]. If this is so, then the virtual absence of BCG from normal bladder epithelium can well be explained by the assessed absence of fibronectin from apical surface epithelium [22]. However, this glycoprotein, being present in plasma, extracellular matrices and basal laminae, may be held responsible for the adherence of BCG observed in the coagulation lesions, since antibodies to fibronectin inhibit the attachment of BCG at these sites in vivo [27]. Alternatively, soluble fibronectin – or an analogue – might inhibit the adherence of BCG by occupying binding sites on the bacterial cells. Moreover, it cannot be excluded in theory that, despite precautions, the SEM preparation procedure itself might be responsible for the possible detachment of mycobacteria if no firm adherence had been accomplished.

The SEM and TEM studies on the interaction of BCG with the T24 human bladder tumor cells in vitro reported here have revealed that BCG-strain mycobacteria readily adhere to and are phagocytized by T24 cells. Nothing is known about the nature of this adherence, but it is undoubtedly a prerequisite for the internalization of the mycobacteria. Morphologically, BCG was internalized by conventional phagocytosis. The time-sequence TEM study revealed that the mycobacteria, once inside the T24 cells, were gradually and progressively degraded in phagolysosomes, formed by fusion of lysosomes with BCG-containing phagosomes.

The ultrastructure of degradative alterations of BCG in T24 cells is largely similar to those seen in the killing and degradation of *Mycobacterium leprae* in skin macrophages of untreated lepromatous patients [5, 31] or in activated mouse macrophages [30], and especially so of *M. aurum* inside mouse macrophages [32]. Silva et al. [32] described five characteristic steps in the degradation process of *M. aurum* in macrophages that could also be recognized in the present study to occur with BCG. It also included the final stage of empty collapsed cell wall remnants representing the most refractory mycobacterial structure (Fig. 2e), and the rather slow course of the clearance.

Data on the intracellular degradation of mycobacteria are rather scarce. Generally, pathogenic species within the genus *Mycobacterium* show different methods of intracellular survival in macrophages, ranging from escape from the phagosome, or prevention of phagosome-liposome fusion, to survival in phagolysosomes due to resistance to lysosomal enzymes [18]. It is probably the mycobacterial cell wall composition and constitution, which is mainly responsible for this resistance [29]. BCG normally also

withstands intracellular degradation [12, 20]. Therefore, it was surprising to find that a supposedly non-phagocytic cell, the human bladder carcinoma cell T24, not only phagocytizes but also kills BCG mycobacteria in vitro. There have been other reports on phagocytosis, but not killing, of mycobacteria by non-phagocytic cells, and recently Ratliff's group also reported observations on the internalization of BCG by T24 cells and MBT-2 (mouse bladder tumor) cells in vitro [1]. They prudently interpreted their data supporting the capacity of these cells to degrade BCG as suggestive. The results presented here strongly support this suggestion.

In some studies [2, 9, 23, 33] it has been observed that intravesical BCG treatment induces MHC class II antigen expression on normal urothelium and bladder tumor cells in patients. BCG can also modulate MHC class II antigen expression on macrophages [10, 19]. Therefore, the expression of this antigen on the phagocytizing T24 human bladder carcinoma cells in vitro was studied. It was found that BCG did not induce an increased class II expression, while IFN gamma did. From the results it was concluded that the increase of MHC class II antigen expression in patients is induced by lymphkokines, including IFN, which can be measured after BCG instillation, and not upon direct contact of tumor cells with BCG.

With the examination of urine sediments and bladder washings from bladder cancer patients undergoing intravesical therapy, we hoped to confirm and extend the obervations with regard to the in vivo and in vitro interactions between BCG and urothelial (tumor) cells. However, cytological and ultrastructural investigations of 682 cells with obvious urothelial morphology, including tumor cells, from a large portion of the samples never showed ingested or partly degraded BCG, while BCG whenever present was phagocytized very clearly by neutrophilic PMNs. The non-involvement of the urothelial cells from these samples in BCG uptake and degradation, as assessed in the present study, is at odds with the results recently published by Ratliff's group [1]. They have found urothelial cells showing ingested and degraded BCG in a few patients, particularly in bladder washing samples obtained 7 days after the instillation. We have not been able to confirm these observations in a more eleborate set of samples.

Our finding of large numbers of PMN leukocytes, especially in the postinstillation urine sediment and bladder washing samples is in agreement with cytofluorometric analyses [7]. Generally, neutrophilic PMNs are the first cells to arrive in response to BCG inoculation or most other bacterial infections, and these cells have been shown to be capable of killing *M. tuberculosis* [3], and probably other mycobacteria as well. Possibly, such a reaction is a starting point for the cascade of events ultimately leading towards the antitumor activity of BCG.

In conclusion, the studies reported here suggest that a direct morphological relationship between BCG and normal bladder epithelium in vivo appears rather unlikely, but an interaction of some BCG product(s) with the bladder wall cannot be excluded. In the light of the negative results with regard to the morphological evidence of an in vivo interaction between BCG and bladder urothelium, it is difficult to assess the relevance of the in vitro finding of phagocytosis and degradation of BCG by the human T24 bladder carcinoma cell line. Further studies are needed for better definition of the nature of the interaction of BCG with the bladder wall.

Acknowledgements. We thank G. Riool-Nesselaar and J. C. Strootman for excellent technical assistance, J. Baelde, N. Hastrich, M. G. Teunissen, I. de Vries and R. E. M. de Vries for their contributions to these studies, Dr. J. G. Vos for critical reading of the manuscript, and C. A. van Maarseveen and C. Muts for secretarial assistance.

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