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The immunomodulatory effects of urine from patients with superficial bladder cancer receiving intravesical evans BCG therapy

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Summary. Bladder cancer cells were stimulated with urine obtained from patients with superficial bladder cancer who had received treatment using intravesical bacillus Calmette-Guérin (BCG). The urine from the first 12 h following each of six BCG instillations was collected and examined for its biological effect. We evaluated effects that had previously been attributed to cytokines detected in the urine of such patients. The modulation of MHC class II antigen and intercellular adhesion molecule-1 (ICAM-1) expression were studied. Using neutralizing polyclonal antibodies to interferon γ and tumour factor α the relative contribution of these molecules to the effects investigated were determined. When cells were stimulated for up to 48 h with first-instillation urine, little effect was seen in any of the parameters investigated. Urine from the sixth instillation, however, proved to be a potent immunomodulatory agent, inducing MHC class II molecule and ICAM-1 expression. Urine from instillations two to five mediated increasing immunomodulatory effects. When sixth-instillation urine samples were treated with neutralizing antibodies to interferon γ prior to their addition to the bladder cancer cells, a marked and significant decrease in their potency was observed. Only in urine from one patient did any immunomodulatory capability remain after antibody treatment. Neutralizing antibodies to tumour necrosis factor α, however, failed to reduce the ability of any patient's urine to induce ICAM-1 expression. When both antibodies were used simultaneously no further decrease in potency was observed. These studies demonstrate for the first time the potential immunomodulatory and cytotoxic effects of urine produced by patients receiving intravesical BCG. Furthermore, in all samples tested, the major immunomodulatory component was shown to be interferon γ. Although tumour necrosis factor α is produced as a result of BCG therapy, this cytokine did not appear to contribute to the parameters investigated. namely the induction of HLA class II antigens, and cell-surface ICAM-1.

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Key words: Bladder cancer – ICAM-1 – MHC class II – BCG therapy – Interferon γ

Introduction

Local immunotherapy with intravesical BCG (bacillus Calmette-Guérin) now has a major role in the treatment and prophylaxis of superficial bladder cancer [13]. Whilst the exact mechanisms of action are poorly understood, findings indicate that this form of therapy is indeed generating an effective host-mounted immune response [18].

When superficial bladder cancer is treated with intravesical BCG, an infiltration of immunologically active T cells into the bladder wall is observed [3] and several cytokines are detected at high concentrations in the urine. For example, interleukin-1 (IL-1), IL-2, tumour necrosis factor α (TNFα) and interferon γ (IFNγ) are transiently produced following BCG instillation [1, 6, 17]. Some controversy exists about the presence of IFNγ in the urine following BCG therapy. Whilst we have detected it at levels in excess of 100 U m/l urine [17], others have failed to detect this molecule despite meticulous care in sample preparation and several methods of assay (A. Bohle, personal communication).

The effects of cytokines on bladder cancer cells have been partially evaluated in vitro with the use of recombinant molecules. Previously we have demonstrated that therapeutically achievable concentrations of IFNγ can induce and augment the expression of MHC class II molecules (DR, DP and DQ) [7]. Furthermore, intercellular adhesion molecule-1 (ICAM-1) expression can also be increased on the surface of established bladder cancer cells following stimulation with IFNγ or TNFα [10, 11]. IFNγ has been shown to exert cytostatic and cytotoxic effects on these same cell lines [8]. TNFα also can exert antiproliferative effects on bladder cancer cells but to a lesser extent than IFNγ (manuscript submitted).

In vivo, following BCG instillation, the new expression of MHC class II molecules by the tumour epithelium has been reported [16]. However, the exact in vivo role of new MHC class II expression and elevated ICAM-1 expression remains to be delineated. These molecules are important to the specific and non-specific immune response. MHC class II molecules are fundamental to the presentation of antigen to CD4+ T cells [12]. Their existence on urothelium does not mean these cells function as antigen-presenting cells; however, studies have shown that MHC class II molecules on vascular endothelium conferred the ability to interact with lymphocytes [15].

ICAM-1 is one of three characterized ligands for the β2 integrin LFA-1 (leucocyte-function-associated antigen-1) [2, 4, 19]. LFA-1 is composed of two trans-membrane chains, an α chain (CD11a) and a β chain (CD18) [14]. ICAM-1 is a cytokine-inducible cell-adhesion molecule found on the surface of many cell types including bladder cancer cells [10, 11]. Our earlier studies have shown that ICAM-1 may serve as a receptor for lymphokine-activated killer (LAK) cells. These observations indicate that binding via ICAM may be a prerequisite for lethal-hit delivery by LAK cells. Therefore, in vivo, it is possible that tumour-infiltrating lymphocytes also use the LFA-1/ICAM interaction as a means of contact with their target cell.

These studies were undertaken to ascertain whether the mixture of cytokines detected by immunoassay techniques in the urine of BCG-treated patients is biologically active. They also provide confirmatory evidence for the excretion of IFNγ in urine of such patients and suggest a possible parameter for predicting therapeutic response to this kind of treatment.

Materials and methods

Cell lines. Three human transitional-cell carcinoma lines RT4, RT112, MGH-U1, supplied by Dr. J. Masters, Institute of Urology, London, were used as a tissue-culture model of bladder cancer. These cell lines represent histopathological tumour grades 1, 2, and 3 respectively. The cell lines have proved to be stable and their cytological appearances and growth characteristics have remained representative of the parent tumours.

The cell lines were routinely grown in RPMI-1640 medium (Gibco, Uxbridge, UK) supplemented with 5% fetal calf-serum (Sera-Lab, Crawley Down, UK), sodium pyruvate (5 mM) and L-glutamine (2 mM). For routine purposes and analysis, cells were recovered by trypsinization (trypsin/EDTA, 0.5 g/l trypsin and 0.2 g/l disodium EDTA) for 4 min.

Patient details. Four patients with superficial bladder cancer were treated with six weekly instillations of intravesical Evans BCG vaccine (formerly Glaxo strain). Each instillation consisted of $(1-5)\times10^9$ colony-forming units of BCG in 60 ml saline.

Preparation and storage of patients' urine. Urine from the 12 h following each BCG instillation was collected and processed within 12 h. Cellular debris was removed by centrifugation at 2000 rpm for 10 min. The cleared sample was then dialysed through a 10-kDa membrane against several changes of phosphate-buffered saline (pH 7.2) overnight at 4° C. The sample was finally filtered through a 0.2 μm-pore filter and stored in aliquots at −70° C.

Cytokines. Highly purified recombinant IFNγ was purchased from Boehringer Mannheim (Lewes, UK) and contained 2.5×10^7 U/mg protein and less than 2 endotoxin U/mg. Highly purified recombinant TNFα was also purchased from Boehringer Mannheim and contained 1.8×10^7 U/mg protein.

Polyclonal and monoclonal antibodies. Neutralising sheep polyclonal antibodies to human IFNγ and TNFα were kindly provided by Dr. A. Meager, (Potters Bar, UK). The murine monoclonal antibodies to human ICAM-1 were generously provided by Dr. T. A. Springer, Harvard Medical School, USA; this antibody is coded RR1/1 and is of the IgG1 isotype. Antibodies to HLA class II molecules were purchased from Dako (High Wycombe, UK). Sheep anti-(mouse-IgG) conjugated to fluorescein isothiocyanate (FITC) was obtained from Sigma (Poole, UK).

Experimental strategy. Dialysed urine was diluted in complete medium prior to application to cells growing in mid log phase in 24-well tissue-culture plates. Control results represent those achieved using dialysed normal urine at the appropriate dilution in RPMI-1640 medium. Samples for neutralising experiments were used at a 1 : 4 dilution. These samples were incubated at 20° C with a 1 : 100 dilution of neutralising antibodies for 1 h prior to stimulation of cells. Controls for neutralization experiments included a dilution of recombinant cytokine in the presence or absence of neutralizing polyclonal antibodies.

Quantification of surface antigen expression. Single-colour immunofluorescence was performed on bladder cancer cell lines stained indirectly with monoclonal antibodies. For each analysis 5×10^5 cells were washed with washing buffer phosphate-buffered saline, 1% fetal calf serum (PBS, 1% FCS, 0.01% sodium azide) and incubated at 4° C for 30 min with optimal concentrations of primary monoclonal antibody. Following two washes, the binding of unconjugated monoclonal antibodies was detected by incubating with a $F(ab')_2$ fragment of sheep anti-(mouse IgG)-FITC conjugate for a further 30 min at 4° C. The cells were washed twice more and resuspended in 1% formaldehyde prior to analysis using a flow micro-fluorometer. Non-viable cells were gated out of the window and at least 5000 events were accumulated using logarithmic amplification of fluorescence intensity.

Determination of IFNγ concentration. The presence of IFNγ in urine samples was analysed using an immunoradiometric assay kit (Medgenix, High Wycombe, UK). Briefly, monoclonal antibodies to human IFNγ are attached to plastic tubes. Following incubation with standards, controls and samples, bound IFNγ is detected with a second antibody to IFNγ labelled with ^{125}I. The radioactive content is assessed using a gamma counter. The lower limit of detection was 0.2 U/ml

Statistical analysis. Statistical analysis was performed using the non-parametric Mann Whitney *U*-test provided in Statview 512 software and using a Macintosh IICX computer.

Results

Modulation of ICAM-1 expression

Previously we have demonstrated that bladder cancer cells constitutively express either ICAM-1 or ICAM-2 molecules on their surface [11]. Furthermore, when these cells were stimulated with recombinant IFNγ, the level of ICAM-1 expression was markedly augmented [10]. In this study we have examined the effect of urine from patients receiving BCG therapy on the expression of ICAM-1.

Urine from the first BCG instillation failed to induce or augment ICAM-1 expression by any bladder cancer cell line (Fig. 1). Only in one patient did first-instillation urine marginally increase ICAM-1 expression. When cells were stimulated for 24 h with sixth-instillation urine a signifi-

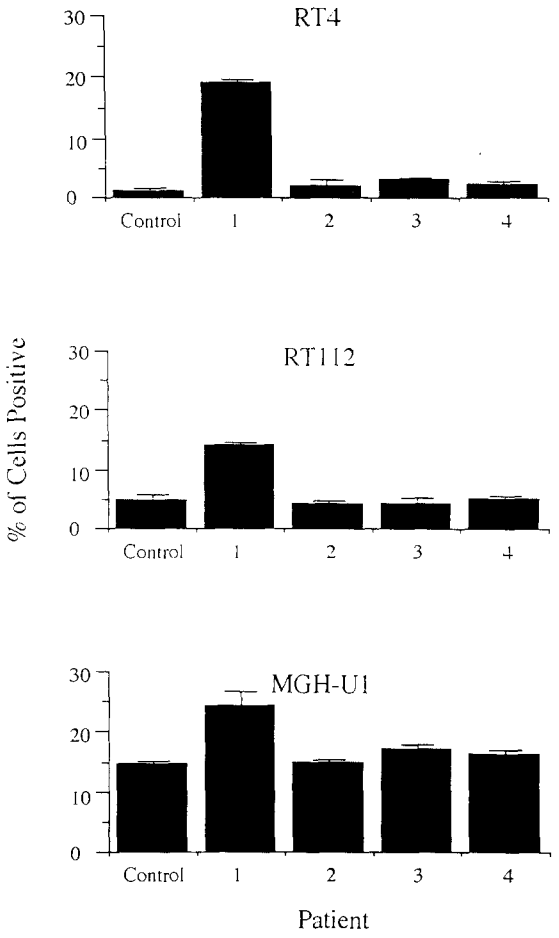

Fig. 1. The effect of urine collected for 12 h after the first instillation of BCG on the expression of ICAM-1 by bladder cancer cells. Three cell lines were stimulated for 24 h in the presence of dialysed urine, following which the percentage of cells expressing ICAM-1 was studied using flow cytometry. *Error bars* show 1 standard deviation

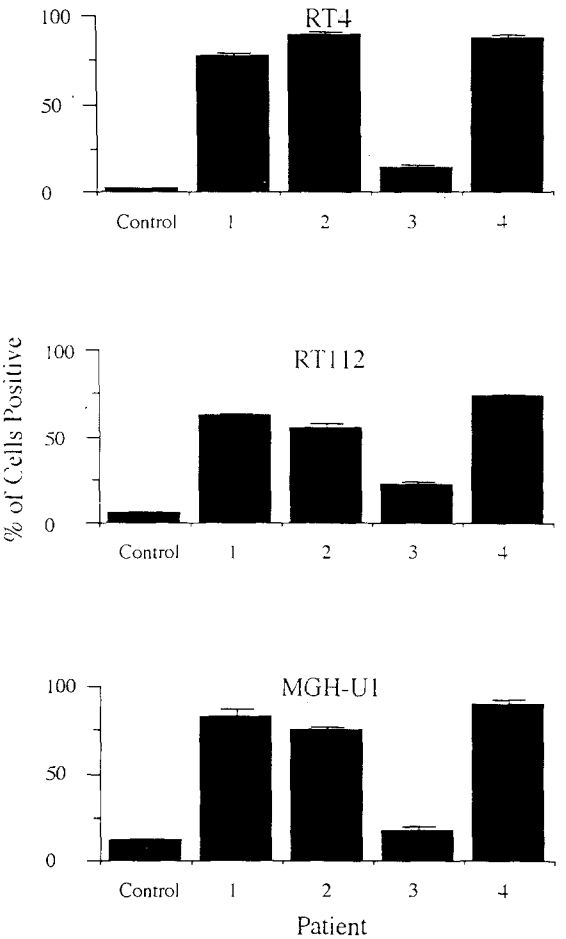

Fig. 2. The effect of urine collected for 12 h after the sixth instillation of BCG on the expression of ICAM-1 by bladder cancer cells. Cells were stimulated for 24 h in the presence of dialysed urine, following which the percentage of cells expressing ICAM-1 was studied using flow cytometry. *Error bars* show 1 standard deviation

cant (P <0.001) increase in ICAM-1 expression was observed on all three cell lines (Fig. 2). The increase was not identical for all the patient samples tested, patient 3 urine only stimulated a small increase in ICAM-1 expression when compared to patient 1. The immunomodulatory effects of urine from BCG-treated patients were apparent at dilutions as high as 1:32 (data not shown). The effect of urine from each of the six instillations is shown in Fig. 3. Maximal response was observed with samples obtained from the latter treatments.

Modulation of MHC class II expression

Previously we have demonstrated that some of the cytokines detected in BCG urine can induce the new expression of MHC class II molecules. As in the case of ICAM-1 expression, urine from the sixth instillation of BCG also induced MHC class II expression (Fig. 4). Urine from the first instillation, however, did not induce or augment class II antigen expression. A graded response was observed for the other instillations.

Controls for neutralizing experiments

Bladder cancer cells were stimulated with recombinant IFNγ and TNFα for 24 h in the presence of absence of neutralizing antibodies to both these cytokines. When neutralizing antibodies were used at a 1:100 dilution they totally abolished the effect of these cytokines on ICAM-1 expression (Fig. 5). This dilution of antibody was sufficient to neutralize at least 1000 U/ml of both cytokines, a level far greater than that detected in the urine following BCG instillation.

Neutralizing antibodies to IFNγ but not TNFα abolish the immunomodulatory effects of urine

When urine from sixth-instillation samples was pre-incubated with neutralizing antibody to IFNγ its ability to modulate ICAM-1 expression was almost completely abolished (Fig. 6). Only in one patient sample did a significant immunomodulatory ability remain. When samples were pre-incubated with neutralizing antibodies to TNFα no significant decrease in ICAM-1 expression was ob-

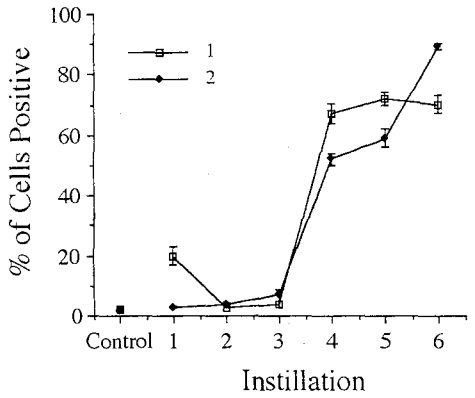

Fig. 3. The effect of urine from six sequential instillations of BCG on the expression of ICAM-1 by RT112 bladder cancer cells. RT4 cells were stimulated for 24 h in the presence of dialysed urine, following which the percentage of cells expressing ICAM-1 was studied using flow cytometry. *Error bars* show 1 standard deviation

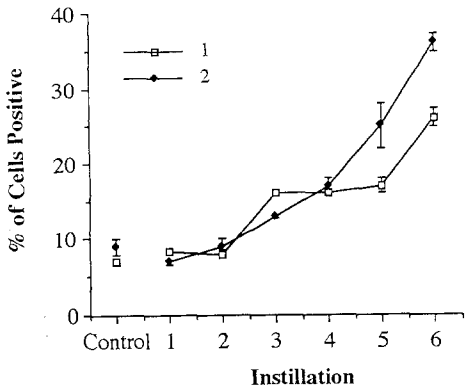

Fig. 4. The effect of urine six sequential instillations of BCG on the expression of MHC class II antigens by RT112 bladder cancer cells. RT4 cells were stimulated for 48 h in the presence of dialysed urine, following which the percentage of cells expressing class II molecules was studied using flow cytometry. Control values represent constitutive class II expression. *Error bars* show 1 standard deviation

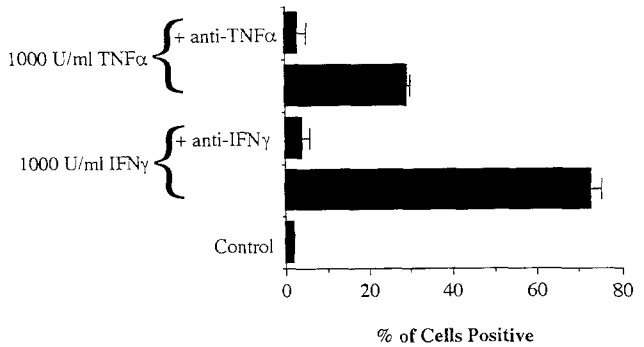

Fig. 5. Control experiment for the neutralization of interferon γ (*IFNγ*) and tumour necrosis factor (*TNFα*) by polyclonal antibodies. RT4 cells were stimulated for 24 h in the presence of recombinant cytokines and neutralizing antibodies, following which the percentage of cells expressing ICAM-1 was studied using flow cytometry. Control values represent constitutive ICAM-1 expression. *Error bars* show 1 standard deviation

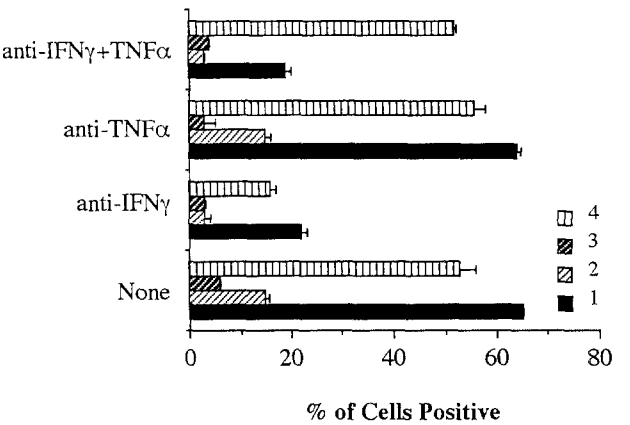

Fig. 6. Neutralization of cytokines in urine from four patients (1–4) from the sixth instillation of BCG using polyclonal antibodies. RT4 cells were stimulated for 24 h in the presence of urine and neutralizing antibodies to IFNγ and/or TNFα. The percentage of cells expressing ICAM-1 was studied using flow cytometry. Control values represent ICAM-1 expression in the absence of neutralizing antibodies. *Error bars* show 1 standard deviation

Table 1. The detection of interferon γ (IFNγ) in the urine of patients during the 12 h following the sixth instillation of BCG, and the total production of IFNγ by each patient during the same 12-h period[a]

Patient	IFNγ concentration (U/ml)	Total IFNγ output during 12 h post BCG (U)
1	24	33720
2	14	6020
3	1.5	1755
4	>90	>46800

[a] Interferon concentration was determined using immunoradiometric assay

served. Incubation with both antibodies simultaneously had no greater effect than with anti-IFNγ antibodies alone.

Detection of IFNγ in the urine of patients following BCG therapy

Using the two-site immunoradiometric assay the presence of IFNγ in urine was confirmed. Peak levels as high as 90 U/ml urine were detected in the later samples. If the volume of urine produced during the 12 hours after instillation is considered, then total IFNγ production can be assessed. Table 1 shows the concentration of IFNγ in the urine and the total IFNγ production during the first 12 h following the sixth instillation of BCG. It should also be noted that IFNγ in the urine of patient 3 never exceeded 1.5 U/ml whilst the other patients secreted levels in excess of 14 U/ml.

Discussion

Intravesical BCG therapy for superficial bladder cancer is the most successful form of immunotherapy for any solid

human malignancy [9]. The mechanisms of action, however, remain largely undetermined. Following repeated BCG instillation, high levels of certain cytokines are readily detected in the urine, whereas patients receiving intravesical chemotherapy do not secrete cytokines [1, 6, 17]. It is likely that these cytokines are involved in the antitumour response of the host. When bladder cancer cells are treated with the recombinant versions of the cytokines detected in the urine they respond in a variety of ways: they express MHC class II and ICAM-1 antigens and their growth characteristics are altered [7, 8, 10, 11].

Although the effects of pure recombinant cytokines have been determined on bladder cancer cells, the effects of the "cocktail" of cytokines present in patients' urine have not. Using dialysed urine we have studied the effect of urine on pertinent immunological parameters. This study confirms the existence of IFNγ in the urine by the use of neutralizing antibodies in a biologically relevant system.

Earlier work from our laboratory has shown that the level of cytokines secreted during the first 12 hours following BCG instillation increases with repeated instillation [17]. In this study we have demonstrated that the biological response to urine increases in a similar manner and our observation leads us to believe that this is largely attributable to IFNγ, First, neutralizing antibodies to IFNγ partially or completely inhibited the induction of ICAM-1 and MHC class II molecules on the surface of bladder cancer cells. Furthermore, using immunoassay, the presence of IFNγ in the urine samples was confirmed. Although substantiating our earlier observations these findings conflict with those of others. A possible reason for this is in the method of the sample preparation. IFNγ is acid-labile and, when present in the low-pH environment of urine (pH 5.5–6.2), its half-life would be shortened. In the study reported by Bohle, urine was neutralized to pH 7.0 using sodium hydroxide [1]; in our study we dialysed urine samples against phosphate-buffered saline (pH 7.2). It is possible that sodium hydroxide altered the structure of IFNγ in some way, thus rendering it immunologically undetectable. Standardization of sampling and detection techniques would be useful as would an agreed expression for the secretion of cytokines. We propose that total cytokine output the first 12 hours following BCG therapy would be a valuable way of presenting such data.

Although several cytokines including IL-1 and TNFα modulate the expression of ICAM-1 and MHC class II molecules by bladder cancer cells (manuscript in preparation) the most potent cytokine in this respect appears to be IFNγ [7]. In the urine of patients receiving BCG therapy this also appears to be the case, as neutralization of IFNγ with antibodies abolished the majority of the effect. Neutralization with antibodies to TNFα, however, did not affect the response of bladder cancer cells. This does not indicate that TNF production is irrelevant to a successful response as it may well be involved in parameters other than those investigated in this study. The samples that gave the least biological response had the lowest levels of immunologically detectable IFNγ, further implying the importance of this molecule.

The source of IFNγ and TNFα is likely to be infiltrating helper T cells and macrophages, increased numbers of which are observed following BCG instillation [3]. Recombinant IFNγ is under clinical evaluation of superficial bladder cancer [5]. Preliminary data, however, fail to demonstrate any advantage over intravesical BCG therapy. Furthermore, the cost of recombinant cytokine therapy is considerably greater than a 6-week course of BCG therapy, which costs around £ 600 per patient. Further studies should evaluate the immunological response to intravesical cytokine therapy and the stability of cytokines such as recombinant IFNγ in urine. Currently we are attempting to dissect the in vivo relevance of MHC class II and ICAM-1 for immunotherapy of bladder cancer. Further studies involving in situ hybridization techniques are also required to divulge the exact cellular source of IFNγ and TNFα. It remains to be determined whether urinary IFNγ may be a prognostic indicator for therapeutic response to intravesical BCG therapy.

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