

New Monoclonal Antibodies against the Putative Immunosuppressive Site of Retroviral p15E

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ABSTRACT

Both retroviral infections as well as human tumors may cause immunosuppression. One of the factors involved in immunosuppression in patients with squamous cell carcinoma of the head and neck (SCC-HN) is a protein related to the retroviral protein p15E. A conserved, 17-amino acid sequence represents the immunosuppressive epitope of retroviral p15E.

In order to study the relationship between SCC-HN associated immunosuppression and retroviral p15E, we produced three new monoclonal antibodies (MAbs; ER-IS1, ER-IS2, and ER-ISS) directed against the immunosuppressive synthetic CKS-17 peptide. These MAbs react with the immunosuppressive peptide (in enzyme-linked immunosorbent assay), with human tumor cell lines (in FACScan analysis), with retroviral p15E (on Western blot), and with cryostat sections of SCC-HN tumor tissue. In addition, the MAbs neutralize the immunosuppressive low molecular weight factors present in sera of patients with SCC-HN.

These results show that retroviral p15E and the immunosuppressive factors associated with SCC-HN share a conserved immunosuppressive epitope and that MAbs against this epitope can be used for detection and neutralization of the tumor-associated immunosuppressive protein(s).

INTRODUCTION

Retroviral infections and virus-induced tumors in both humans and animals are often accompanied by the development of immunosuppressive effects (1, 2). The env gene-encoded surface and transmembrane proteins of retroviruses are responsible for these effects (3).

P15E is a hydrophobic transmembrane protein of approximately M_r 19,000 present in all transforming type C retroviruses (4).

The immunosuppressive activities of retroviral p15E have been thoroughly studied *in vivo* and *in vitro*. Isolated p15E of feline leukemia virus inhibits *in vivo* tumor immunity, and murine leukemia virus p15E inhibits macrophage accumulation at inflammatory foci in mice. *In vitro*, p15E causes inhibition of lymphocyte proliferation, inhibition of interleukin 2 secretion, and monocyte chemotaxis. (for reviews, see Refs. 3 and 5).

The antiinflammatory activities of p15E may, in general, contribute to the pathogenicity of retroviruses. P15E might also be responsible for part of the immunosuppression associated with retroviral infections (5) or virus-induced tumors.

Proteins physicochemically related to retroviral p15E have been identified in (virus-free) murine malignant cell lines as well as in human malignant cell lines, malignancies, and cancerous effusions. These p15E-related proteins also have immunosuppressive properties (6-8).

Especially patients with SCC-HN² manifest defects in their cell-mediated and humoral immune function (9, 10). Retroviral p15E-

related immunosuppressive factors were identified in serum obtained from patients with SCC-HN (11) and can be detected in tumor tissue of SCC-HN (12, 13).

The exact molecular nature of these p15E-related immunosuppressive factors has not yet been clarified. Immunosuppressive LMWFs isolated from human tumors show cross-reactivity with monoclonal antibodies directed against retroviral p15E (14).

In retroviral p15E, the putative immunosuppressive site is located in a highly conserved 26-amino acid region of the protein (15). A synthetic peptide, CKS-17, corresponding to 17 amino acids selected from this region (conjugated to a carrier protein; Ref. 16), has similar immunosuppressive actions as the intact retroviral p15E protein (17-20).

In this study, we investigated whether p15E-related immunosuppressive proteins expressed in or on human tumor cell lines or in tissue specimen of human SCC-HN contain an epitope similar to the conserved CKS-17 epitope. For this purpose, we applied peptide specific MAbs generated against the immunosuppressive CKS-17 epitope.

Furthermore, we investigated whether immunosuppression by LMWFs in sera from patients with tumors expected not to be virally induced *e.g.*, SCC-HN, is caused by proteins containing this immunosuppressive CKS-17 epitope.

We found that our MAbs react with human tumor cell lines and with antigens from cells of human SCC-HN. Of special interest is the fact that the antibodies block the immunosuppressive activity of LMWFs present in serum of patients with SCC-HN.

MATERIALS AND METHODS

Cell Lines. U937 is a human histiocytic lymphoma cell line (21) which produces p15E-related immunosuppressive factors (8). Cells were cultured in RPMI 1640 containing 5% FCS, 100 μ g/ml penicillin, and 60 μ g/ml streptomycin (P/S).

P2 is a human squamous cell carcinoma cell line isolated from a patient with lung carcinoma (cells were obtained from Dr. L. de Ley, University Hospital, Groningen, the Netherlands). Cells were cultured in RPMI 1640 containing 15% FCS, 0.05 mM β -mercapto-ethanol, 2 mM glutamine, 1 mM sodium-pyruvate, and P/S.

JLS-V5, a Rauscher MuLV producing cell line (22), was used for isolation of MuLV. The cells were cultured in Dulbecco's modified Eagle's medium containing 7% FCS and P/S. Isolation of MuLV was performed according to Duesberg and Robinson (23).

Peptide Synthesis and Conjugation. CKS-17 peptides were synthesized using solid phase synthesis as described originally by Merrifield in 1963 (24). Purification of peptides was performed by gel filtration and reverse phase high performance liquid chromatography. A cysteine residue was added to the amino-terminus during synthesis.

Peptide conjugates for immunizations were prepared by coupling peptides to KLH by means of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described in detail by Van Denderen *et al.* (25). Peptide conjugates for ELISA experiments were produced by coupling to BSA by means of EDC.

The synthesized peptide consisting of the CKS-17 amino acid sequence (LQNRRLDLLFLKEGGL) (16) is hereafter called SP124. A shorter peptide, lacking the first 6 amino-acids (LDLLFLKEGGL), is named SP125.

SP124 coupled to KLH via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide

Received 8/4/93; accepted 2/2/94.

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² The abbreviations used are: SCC-HN, squamous cell carcinoma of the head and neck; LMWF, low molecular weight factors; MAbs, monoclonal antibodies; FCS, fetal calf serum; P/S, 100 μ g/ml penicillin and 60 μ g/ml streptomycin; MuLV, Murine leukemia virus; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; TGF, transforming growth factor.

ester is referred to in this report as SP124-KLH, and peptides coupled to BSA via EDC are referred to as SP124-BSA and SP125-BSA.

Production and Purification of Monoclonal Antibodies. Female, 12-week-old BALB/c mice were immunized s.c. on the upper side of both hind feet with 50 μ g of SP124-KLH in complete Freund's adjuvant (26). An i.p. booster injection was given 4 weeks later with 50 μ g SP124-KLH in incomplete Freund's adjuvant. Popliteal lymph node cells were isolated 3 days later for fusion with mouse SP2/0-Ag14 myeloma cells using polyethylene glycol (PEG 4000; 72%). Cell fusion and subcloning of positive hybridomas were performed under standard conditions. Selection was performed with 1 μ g/ml azaserine (27) in the presence of 40 units/ml interleukin 6.

For large scale production of MABs, 1×10^8 hybridoma cells were cultured in 1.5 liters RPMI 1640 containing 5% FCS and P/S for 10 days in gas-permeable Fenwal Lifecell bags (Baxter). MABs were purified using a protein-A-Sepharose column. IgM antibodies were purified using rat-anti-mouse- κ antibodies coupled to Sepharose beads (28).

ELISA Experiments. Micro-ELISA experiments were performed using Terasaki microtiter trays and β -galactosidase-labeled sheep-anti-mouse antibodies (Amersham; Ref. 29). For the determination of immunoglobulin, production wells were coated overnight at 4°C with 10 μ l of 10 μ g/ml 226-anti- κ antibodies (28). Specific binding of MABs to synthetic peptides was determined using wells coated overnight at 4°C with peptide-BSA conjugates (0.1 μ g/well) or BSA alone (0.1 μ g/well) as a control. Binding was expressed as arbitrary fluorescence units.

For blocking experiments, antibodies were incubated overnight at 4°C with free synthetic peptide (SP124) in various concentrations before testing the mixture in ELISA. Binding is expressed as a percentage of the binding of the unabsorbed antibody.

Flow Cytometry. For FACScan analysis, U937 and P2 cells were washed in medium without serum and fixed in 2% paraformaldehyde (Merck) in PBS for 10 min at 4°C. Next, the cells were washed three times in PBS and permeabilized with 100% methanol for 20 min at 4°C. After washing the cells twice, the cells were blocked for at least 30 min in PBS-0.1% BSA. Cells ($0.5\text{--}1 \times 10^6$) in a volume of 10 μ l of washing buffer (PBS-0.05% Tween 20 and 0.1% BSA) were incubated with 50 μ l of relevant MAB containing culture supernatant for 10 min at room temperature. After washing the cells four times, a fluorescein isothiocyanate-labeled secondary antibody (rabbit-anti-mouse-fluorescein isothiocyanate; DAKO) was added in an optimal dilution. Incubation was allowed for 10 min at room temperature. Cells were then washed and stored in the dark at 4°C until analysis. MABs directed against irrelevant synthetic peptides (25) were used as control antibodies. Culture supernatant of SP2/0 cells was used to determine background fluorescence. A Becton and Dickinson FACScan was used for flow cytometric analysis.

Gel Electrophoresis and Immunoblotting. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, 15 μ g of lysed MuLV particles were electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred from the gel onto a 0.2 μ m nitrocellulose membrane. After transfer, the nitrocellulose sheet was washed in PBS and submerged in a solution of nonfat milk for at least 30 min to block remaining binding sites. The sheet was cut into strips for individual staining with antibodies. Detection of bound antibody was performed with alkaline phosphatase conjugate antibodies using 5-bromo-4-chloroindoxyl phosphate and nitro blue tetrazolium (30). Culture supernatant of the MAB 19F8, directed against MuLV p15E (IgG2b; Ref. 31) was used as a positive control; antibodies directed against irrelevant synthetic peptides (25) were used as negative control antibodies.

Monocyte Polarization. The monocyte polarization assay, originally described by Cianciolo *et al.* (6), was performed with slight modifications (11). After induction of polarization with *N*-formyl-methionyl, monocytes were counted with a hemocytometer using a light microscope. Two hundred cells were counted and judged individually by two persons for "polarization" as described by Tas *et al.* (32). The capability of the LMWFs of patient sera to inhibit the polarization of healthy donor monocytes was determined as described (32).

Adsorption of Patient Sera with Monoclonal Antibodies. Sera of patients with SCC-HN, which inhibited the above described polarization assay, were incubated with the new MAB supernatant at 4°C for 16h, followed by Amicon ultrafiltration to remove immune-complexes formed. Control adsorption experiments were carried out with a mixture of the p15E-specific anti-

bodies 19F8 and 4F5 (IgG2a; Ref. 7) in a final concentration of 25 μ g/ml each or with the negative control antibody MPC11.UoA (IgG2b; ATCC, Rockville, MD). The adsorbed serum fractions were tested in the monocyte polarization assay.

Immunohistochemistry. Immunohistochemistry was carried out on cryostat sections of SCC of the larynx. Sections (6 μ m) were fixed in acetone for 15 min at 4°C. Sections were incubated with MAB culture supernatant. Bound antibodies were detected with horseradish-peroxidase-conjugated goat-anti-mouse Ig antibodies (Miles). The diaminobenzidine staining procedure was carried out as described in detail elsewhere (33). Sections were weakly counterstained with hematoxylin.

RESULTS

Monoclonal Antibodies React with CKS-17 Peptide and with Human Cell Lines Expressing p15E-related Factors. Fusion experiments resulted in the generation of several hybridomas, primarily selected on the basis of Ig production and reactivity with peptide conjugates.

Fig. 1, A-C shows the reactivity of three monoclonal antibodies, ER-IS1 (IgM), ER-IS2 (IgM), and ER-IS5 (IgG2b), with the peptide conjugates BSA-SP124 and BSA-SP125. The antibodies ER-IS1 and ER-IS5 react with SP124; no cross-reactivity with BSA was detected. The antibody ER-IS2 reacts with the shorter peptide SP125; in addition, a very weak background signal against BSA was detected.

Free SP124 peptide was able to block the reactivity of ER-IS1, ER-IS2, and ER-IS5 with the relevant peptide conjugate in a concentration-dependent manner (Fig. 1D). The reactivity in peptide-ELISA of suboptimal concentrations (10 μ g/ml) of ER-IS1 and ER-IS2 antibodies could be blocked up to 80%, whereas the reactivity of ER-IS5 could be blocked up to 50% of the maximum signal.

To study the reactivity of the antibodies with the human p15E-related protein present in malignant cell lines, we performed FACScan analysis. Cells were fixed and permeabilized to be able to detect antigens located in the cytoplasm of the cells. All three antibodies recognized the immunosuppressive epitope in U937 (histiocytic lymphoma) and P2 (squamous cell carcinoma) cells (Fig. 2). Control antibodies showed fluorescence at background levels (data not shown). No clear positive staining was shown without fixation, indicating the cytoplasmic localization of the immunosuppressive protein(s) similar to p15E-related proteins detected with the MAB 19F8 (6).

Retroviral p15E Is Recognized by the MABs ER-IS1, ER-IS2, and ER-IS5. Reactivity of the new MABs with MuLV-p15E was determined by immunoblotting. The MAB 19F8, directed against MuLV p15E, was used as a positive control. Two distinct bands corresponding to p15E (M_r 19,000) and its degradation product p12E (M_r 17,000; Ref. 7) were detected by 19F8. Fig. 3 shows that the monoclonal antibodies ER-IS1, ER-IS2, and ER-IS5 react specifically with the MuLV-p15E protein; these three antibodies detect the dominant p12E band. Control antibodies against irrelevant synthetic peptides showed no reactivity with retroviral proteins (data not shown).

Antibodies Reduce LMWF-induced Inhibition of Monocyte Polarization. To investigate whether the immunosuppressive epitope was detectable on p15E-related LMWFs in sera of patients with SCC-HN, we performed polarization experiments. We selected patient sera with a high inhibition percentage in the polarization assay (LMWF, 40–60% inhibition) for adsorption experiments with our panel of MABs. Three experiments were carried out (Table 1). After overnight incubation of sera with the MABs directed against CKS-17, monocyte polarization experiments were performed. Inhibition in the polarization assay by serum LMWFs was significantly reduced after preincubation with ER-IS1, ER-IS2, and ER-IS5 at 4°C. The mixture of 19F8/4F5 directed against retroviral p15E causes a comparable

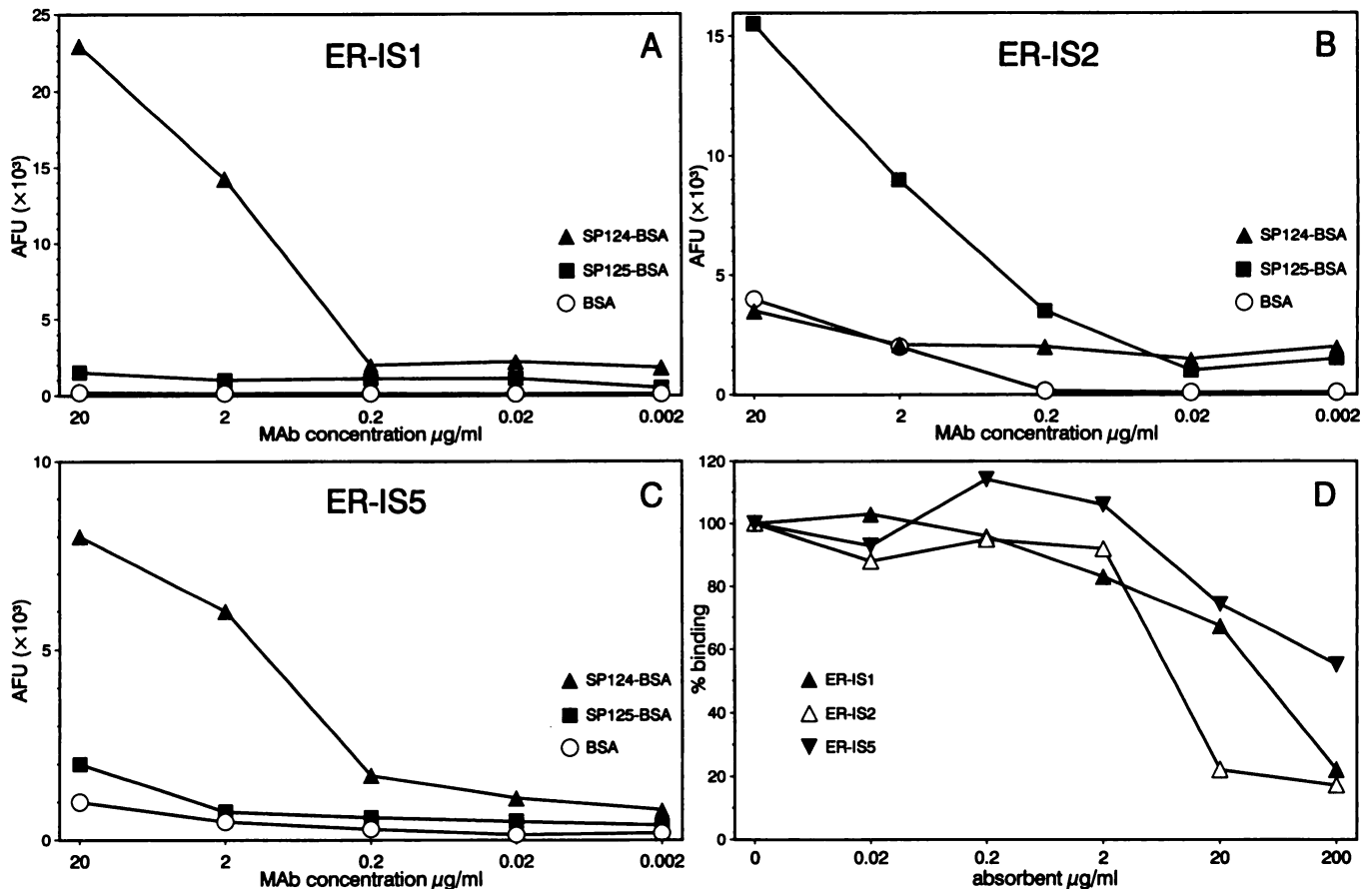


Fig. 1. Binding characteristics of MAbs to SP124-BSA, SP125-BSA, and BSA alone in ELISA (A-C). Serial dilutions of hybridoma culture supernatant with known antibody concentrations were tested for binding to peptide conjugates. Binding is expressed as arbitrary fluorescence units (AFU). D, inhibition of binding after preadsorption with SP124. Antibodies were incubated with various concentrations of SP124 and tested in ELISA for reactivity with SP124-BSA (ER-IS1 and ER-IS5) or SP125-BSA (ER-IS2). Binding is expressed as a percentage of the binding of suboptimal concentrations of unadsorbed MAb.

reduction of inhibition. Incubation with control antibody MPC11 did not reduce inhibition of polarization (Table 1).

Not all the inhibitory activity in the sera could be adsorbed out by incubation with the antibodies. Furthermore, combining the anti-p15E antibodies with the three anti-CKS-17 antibodies could not further reduce the inhibition as compared to the individual antibodies (data not shown). Taken together, these results indicate that the immunosuppressive epitope represented by the CKS-17 peptide is expressed on human p15E-related factors.

Staining of SCC-Tumor Tissue with ER-IS1, ER-IS2, and ER-IS5. In order to investigate the presence of the CKS-17 epitope in human SCC-tumor tissue, we performed immunohistology. Cryostat tissue sections were made from SCC larynx biopsies and stained with monoclonal antibodies.

The MAbs ER-IS1, ER-IS2, and ER-IS5 showed reactivity with human squamous cell carcinoma tissue. The control antibody 19F8 directed against retroviral p15E showed only a weak staining of tumor cells. The antibody 4F5, directed against a different epitope of retroviral p15E (7), showed strong reactivity with the corneal layers of well-differentiated areas of the tumor as was shown before (13).

Antibody ER-IS1 showed a very strong reactivity with well-differentiated areas of the tumor (Fig. 4A). A cytoplasmic staining pattern was observed. However, cells in the basal layer of the epithelium did not react with ER-IS1. No cells in the surrounding connective tissue (fibroblasts, endothelial cells, or lymphocyte infiltrate) were stained, as was shown by comparison with the staining pattern of the control antibody (MPC 11; Fig. 4D).

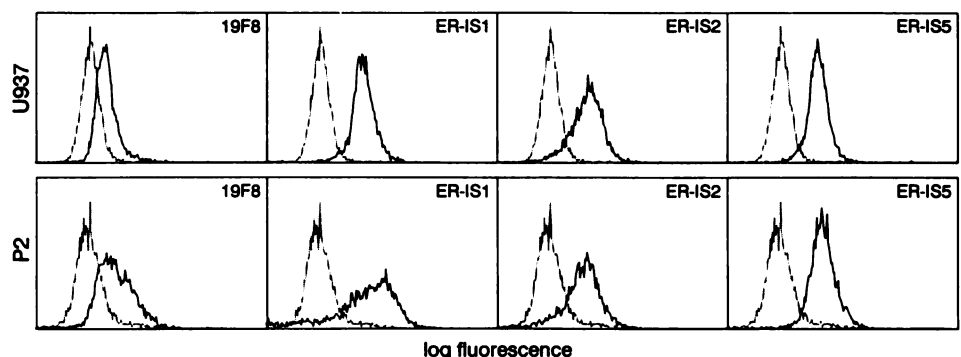


Fig. 2. FACSscan analysis of human tumor cell lines (U937 and P2). Cells were fixed, incubated with MAbs against retroviral p15E (19F8) or against the immunosuppressive epitope (ER-IS1, ER-IS2, and ER-IS5), and analysed. Thin line, background fluorescence.

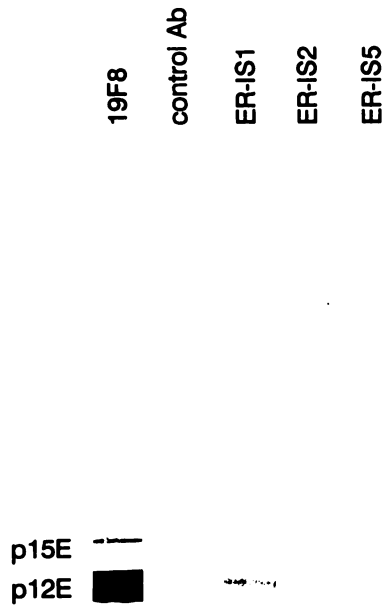


Fig. 3. Western blot of MuLV proteins detected with MAbs against retroviral p15E (19F8), irrelevant control antibody, or antibodies against the immunosuppressive epitope of p15E (ER-IS1, ER-IS2, and ER-IS5).

The antibodies ER-IS2 and ER-IS5 detected all layers of infiltrating tumor in the tissue sections (Fig. 4, B and C). These antibodies also detected antigens in the cytoplasm of the tumor cells, but their staining was less strong than that of ER-IS1. In contrast to the staining with anti-p15E antibodies 19F8 and 4F5 and ER-IS1, with ER-IS2 and ER-IS5 a few cells in the surrounding tissue were stained. These cells could either be single epithelial tumor cells or macrophages involved in the inflammatory processes accompanying tumor growth.

DISCUSSION

Identification and quantitative detection of immunosuppressive proteins associated with human cancer is necessary for a better understanding of the immunological processes involved in human cancer. Knowledge about immunosuppression is of special importance regarding immunomodulatory therapy. The limited success of immunomodulatory therapy could well be explained by the presence of immunosuppressive proteins interfering with therapy. Blocking of these proteins could be helpful in improving the success of immunotherapy, especially for patients with head and neck cancer.

In this paper, we describe three new MAbs which react with the putative immunosuppressive site of retroviral p15E. These MAbs were generated after immunization of mice with an immunosuppressive peptide corresponding to the conserved immunosuppressive site of p15E. This CKS-17 peptide has similar immunosuppressive actions as the retroviral p15E-protein. CKS-17-carrier inhibits human mitogen and alloantigen-stimulated lymphocyte proliferation (16, 34), human natural killer cell activity (35), interleukin 1-mediated monocyte tumor killing (17, 36), interleukin 2 production (19), immunoglobulin synthesis (37), TNF- α mRNA expression, (38) and protein kinase C activity (39, 40). Monocyte chemotactic responsiveness was inhibited by free CKS-17 peptide (20).

Although ER-IS1, ER-IS2, and ER-IS5 all detect p15E-related proteins, their reactivity pattern is not identical. The observed difference in reactivity could be explained by the fact that these MAbs are not directed against exactly the same peptide epitope. In ELISA,

antibodies ER-IS1 and ER-IS5 reacted with the 17-aa peptide SP124 coupled to BSA; ER-IS2 reacted with SP125 coupled to BSA. Conjugation of SP124 and SP125 to BSA was performed through their COOH groups using EDC as an coupling agent. SP124 exposes three COOH groups; consequently, EDC-coupling may result in a mixture of three types of peptide-BSA conjugates, each of which would be capable to form a loop-like conformation in which NH₂- and COOH-terminal ends are in close association. For the 11-aa SP125 conjugate, different loop-like conformations are formed. Therefore, the MAbs could react with one of these different peptide conjugates of SP124 and SP125. Inhibition experiments with free SP124 showed that reactivity of ER-IS1, ER-IS2, and ER-IS5 against peptide conjugates could be blocked for 50–80%. This result proves their specificity for the immunosuppressive epitope, but the fact that the reactivity could not totally be blocked with the linear peptide indicates that only specific conformational epitopes are recognized by the antibodies.

The specificity of ER-IS1, ER-IS2, and ER-IS5 for the immunosuppressive epitope of p15E was further revealed by the detection of MuLV-p15E on Western blot. These MAbs detected the same protein band as the MAb 19F8. However, the reactivity of our new MAbs against retroviral p15E was less strong than the reactivity of 19F8 against p15E. Therefore, only the p12E degradation band was detected with ER-IS1, ER-IS2, and ER-IS5.

MAbs ER-IS1, ER-IS2, and ER-IS5 recognized cytoplasmic p15E-like proteins present in human tumor cell lines as determined by FACScan analysis. Although not all human tumors contain the p15E-related epitope (11), the tumor cell lines presently investigated contain p15E-related proteins as determined with antibodies against retroviral p15E (8). FACS analysis of cell lines with our MAbs reveals the presence of the CKS-17 epitope in the cytoplasm of human tumor cell lines.

In human tumor tissue, the presence of proteins containing the immunosuppressive epitope was shown with immunohistology. However, the staining pattern with the three antibodies was not identical. ER-IS1 recognized more differentiated areas of the tumor, whereas ER-IS2 and ER-IS5 stained all tumor cells. The existence of different proteins expressing the same immunosuppressive epitope in different tumor areas or differentiation stages could be an explanation for these staining patterns. Until now, it is not clear whether there is one p15E-like protein or whether there are more immunosuppressive proteins, all containing (parts of) the immunosuppressive epitope represented by the CKS-17 peptide. Some homologies of CKS-17 with other (immunosuppressive) proteins have already been described. These comprise homology with TGF- β (41) and interferon α (40). However, our preliminary experiments show that α CKS-17 MAbs have a different reaction pattern with cell lines compared to α TGF- β MAb, indicating that these MAbs do not recognize the same protein.

Monocyte polarization experiments confirmed the presence of the immunosuppressive epitope on low molecular weight proteins in sera of patients with SCC-HN. Adsorption experiments with the MAbs against the immunosuppressive epitope showed a significant reduc-

Table 1. Relative inhibition of the fMLP-induced polarization of human monocytes by LMWFs in sera of patients with SCC-HN before and after adsorption with monoclonal antibodies

Expt. ^a (n)	LMWF	ContrAb	Adsorption with			
			19F8/4F5	ER-IS1	ER-IS2	ER-IS5
1 (8)	39 $\pm$ 6 ^b	37 $\pm$ 4	14 $\pm$ 8 ^c	ND	ND	23 $\pm$ 8 ^c
2 (5)	55 $\pm$ 5	50 $\pm$ 12	28 $\pm$ 10 ^c	20 $\pm$ 14 ^c	ND	ND
3 (1)	42	ND	ND	12	21	ND

^a Expt., experiment; ContrAb, control antibody.

^b Values ($\pm$ SD) represent the mean inhibition percentage of the fMLP-induced monocyte polarization in the presence of LMWFs. To investigate whether the MAbs against the immunosuppressive epitope bind to these LMWFs, adsorption experiments were performed using different monoclonal antibodies.

^c $P < 0.005$ (paired Student's t test); ND, not done.

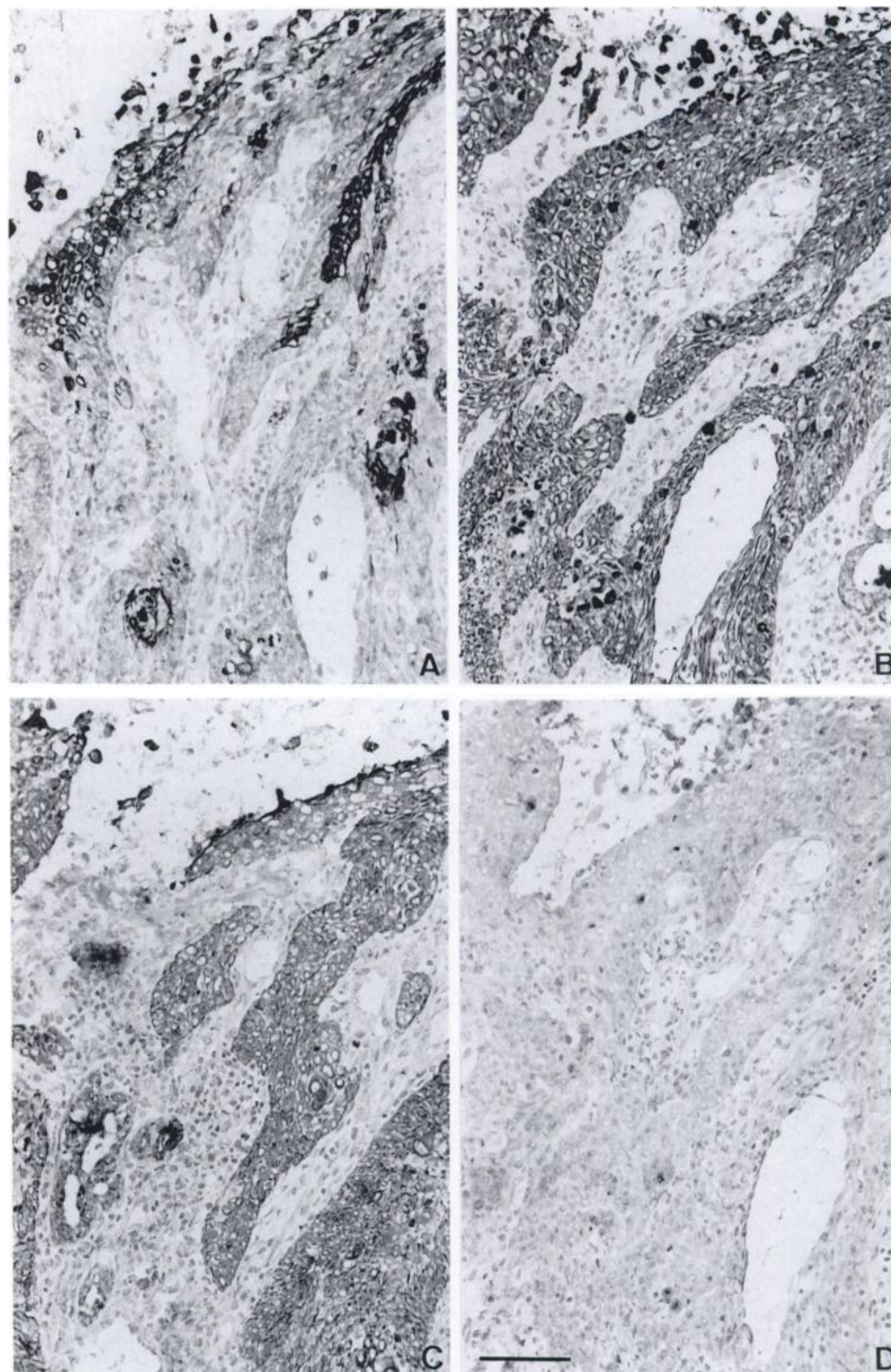


Fig. 4. Immunohistochemical staining of serial cryostat sections of infiltrating SCC of the larynx with monoclonal antibodies. (A, ER-IS1; B, ER-IS2; C, ER-IS5; and D, control antibody). Bar, 100 μ m.

tion of the inhibition in the polarization assay. However, only about 50% of the inhibition could be blocked; combining the anti-p15E with the anti-CKS-17 antibodies did not result in a higher blocking percentage, indicating the presence of other polarization inhibitory molecules of low molecular weight. Observations made in our laboratory show that interferon α could possibly be one of the polarization inhibitory cytokines present in serum of patients with SCC-HN (42). Another polarization inhibitory cytokine present could well be TGF- β , a cytokine which was identified in several tumors associated with immunosuppression (10). Immunoprecipitation and purification experiments are presently being performed in order to reveal the exact nature of the immunosuppressive p15E-related protein(s).

We are planning to use our antibodies directed against a functional immunosuppressive epitope for the quantitative detection of immunosuppressive proteins involved in the development of human cancer. The detection of immunosuppressive molecules could be a helpful tool in the detection of a recurrent tumor because the production of p15E-related molecules is correlated with the growth of a tumor (43).

ACKNOWLEDGMENTS

We thank T. M. van Os for the production of photographs and figures.

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