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ORIGINAL ARTICLE

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Construction and characterization of the chimeric monoclonal antibody E48 for therapy of head and neck cancer

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Abstract Data from an ongoing clinical radioimmunoscintigraphy trial indicate that ^{99m}Tc-labeled monoclonal antibody (mAb) E48 is highly capable of selectively targeting squamous cell carcinoma of the head and neck (HNSCC). The percentage of the injected dose per gram of tumor tissue was found to be high, rendering mAb E48 a promising candidate mAb for therapeutic purposes. We now describe the construction of a chimeric (mouse/human) mAb E48 by recombinant DNA technology. The genes encoding the variable domains of the heavy and light chain were cloned and ligated into expression vectors containing the human γ1 heavy-chain gene and the human κ light-chain gene respectively. Biological properties of the resulting chimeric mAb E48 were compared to the murine form in vitro and in vivo. The reactivities of chimeric (c)mAb and murine (m)mAb E48 with HNSCC, as assessed by immunohistochemical staining as well as immuno-blotting were shown to be similar. The affinity constant appeared to be $0.9 \times 10^{10}\,M^{-1}$ and $1.6 \times 10^{10}\,M^{-1}$ for the mmAb and cmAb respectively. The biodistribution of both antibodies was tested by simultaneous injection into nude mice bearing human HNSCC xenografts. cmAb E48 was found to be cleared more rapidly from the blood than mmAb E48, resulting in a 30% lower tumor uptake but similar tumor to non-tumor ratios, 3 days after injection. Moreover, it was shown that cmAb E48 is highly capable of lysing HNSCC targets in ADCC assays in vitro, whereas the mmAb appeared to be almost inactive. These data indicate that cmAb E48 has potential as a targeting agent for the eradication of HNSCC in man.

Key words Chimeric antibody · Head and neck cancer Squamous cell carcinoma · Radioimmunotherapy Antibody-dependent cellular cytotoxicity

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Introduction

Of all human neoplasms, squamous cell carcinoma (SCC) is one of the most frequent tumor types, representing the major histological type in the upper aerodigestive tract, the genital tract and the skin. The sensitivity of SCC of the head and neck (HNSCC) to irradiation is one of the main reasons to focus on the possibility of using monoclonal antibodies (mAb) directed against tumor-associated antigens for the targeting of radioisotopes to HNSCC tumors [38, 41]. A panel of mAb directed against SCC-associated antigens was selected and one of those, designated mAb E48, recognized a 15- to 20-kDa surface antigen expressed on HNSCC as well as squamous epithelial cells [26–28, 33]. Initial preclinical studies indicated that mAb E48 may be well suited for targeting HNSCC [8]. The feasibility of the approach was demonstrated by regressions and complete remissions of established HNSCC xenografts in nude mice as a result of a single injection of ^{131}I-labeled or ^{136}Re-labeled E48 IgG [9–11]. Selective tumor accumulation of mAb E48 was also observed in a clinical phase I/II radioimmunoscintigraphy trial, in which the safety and diagnostic accuracy of ^{99m}Tc-labeled E48 IgG and $F(ab')_2$ for detection of lymph node metastases in HNSCC patients were evaluated [6, 37]. Measurement of

radioactivity in biopsies revealed that the percentage of the injected dose per gram of tumor was high for mAb E48 IgG in these studies, ranging from 0.014% to 0.082% ($n = 16$) 44 h after injection [38].

When the targeting potential of mAb E48 in HNSCC patients and the therapeutic efficacy of radiolabeled mAb E48 in tumor-bearing nude mice are taken into account, it seems obvious that radioimmunotherapy with E48 IgG may have important potential for the elimination of minimal residual disease [38]. However, administration of high doses of murine mAb (mmAb) to patients usually induces human anti-(mouse antibody) (HAMA) responses, which lead to a rapid clearance of the injected antibody and sometimes to anaphylactic shock [18, 30]. One possible way to avoid HAMA responses is the use of human-mouse chimeric antibodies (cmAb), which can be produced by recombinant DNA techniques [22, 25]. Such cmAb are composed of the antigen-binding variable regions of the murine mAb, fused to the heavy- and light-chain constant regions of the human immunoglobulins. Chimerization is particularly effective to avoid HAMA responses directed against the constant region. For instance, a marked reduction in anti-immunoglobulin response in patients was shown with the use of cmAb 17-1A compared to mmAb 17-1A, whereas the localization of cmAb 17-1A was found to be comparable to that of mmAb 17-1A [20, 24]. For mmAb where the variable region is highly immunogenic, chimerization may be less effective. As recently reported by LoBuglio et al. [21], the chimeric form of B72.3 induced an immune response in 16 out of 24 patients, directed primarily to the murine variable (V) region. When HAMA responses are directed against the murine framework of the variable domain, the problem can sometimes be solved by grafting the complementary determining regions on human frameworks [16]. This technique, however, sometimes results in a serious decrease of the affinity. On the basis of these considerations, we decided to apply the technique of chimerization as a first step in tailoring mAb E48 for therapeutic application.

It is well known that mAbs can cooperate with lymphocytic and monocytic effector cells to kill tumor cells in vitro and in vivo [4, 15, 29, 31]. When constructing cmAb, several investigators have varied the isotype of the cmAb to improve mediation of such antibody-dependent cellular cytotoxicity (ADCC), which may lead to improved clinical efficacy. cmAb having a human IgG1 (γ_1) constant region were shown to be the most active in mediating ADCC [35]. When using such cmAb for radioimmunotherapy of minimal residual disease it can be anticipated that ADCC activity may be supportive to irradiation, especially in the eradication of single disseminated cells or small aggregates.

In the present study the construction of the human/mouse cmAb E48 is described. cmAb E48 was compared with mmAb E48 for biological activities such as antigen recognition, affinity, in vivo biodistribution in HNSCC-bearing nude mice and potential for the mediation of ADCC.

Materials and methods

Restriction enzymes were purchased from Boehringer Mannheim (Boehringer Mannheim B.V., Almere, The Netherlands) or Pharmacia (Pharmacia Biotech Benelux, Roosendaal, The Netherlands). DNA polymerase (Klenow fragment) and T4 DNA ligase were from Boehringer Mannheim, Avian myeloblastosis virus (AMV) reverse transcriptase was from Promega (Promega, Leiden, The Netherlands) and T4 polynucleotide kinase was from Pharmacia. T7 DNA polymerase used for sequencing was from Pharmacia. The reaction conditions were chosen as recommended by the supplier. ^{32}P-labeled nucleoside triphosphates (3000 Ci/mmol) were purchased from NEN (NEN Research Products, Boston, Mass., USA). Sodium [^{51}Cr]chromate (spec. act. 332 mCi/mg Cr) used for ADCC assays was obtained from Amersham (Amersham Nederland B.V., 's-Hertogenbosch, The Netherlands).

Squamous cell carcinoma cell lines

The HNSCC cell lines UM-SCC-22A and UM-SCC-14C used for Western blotting, binding assays and ADCC experiments, were kindly provided by Dr. T. E. Carey (Ann Arbor, Mich.). Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Breda, The Netherlands), 5% fetal calf serum (FCS; HyClone, Logan, Utah, USA), 2 mM L-glutamine, 1% penicillin/streptomycin (Cellect, ICN Biomedicals B.V., Amsterdam, The Netherlands), 16 mM $NaHCO_3$ and 15 mM HEPES, pH 7.4, in humidified air and 5% CO_2 at 37° C. As checked by immunocytochemistry of cytospin preparations, 100% of the UM-SCC-22A cells and none of the UM-SCC-14C cells were shown to express the E48 antigen irrespective of the culture conditions.

Probes

The 2-kilobase (kb) *Eco*RI/*Bam*HI fragment, containing the murine germline Jh3-Jh4 joining region was used to detect the murine heavy-chain genes (mJh probe). The 2.8-kb *Hin*dIII fragment containing the murine germline Jκ region was used for the detection of the murine κ light-chain genes (mJk probe; probes were kindly provided by Dr L. Coney, Centocor Inc., Malvern, USA). For identification of specific Jκ regions the following DNA fragments were used: the 0.8-kb *Hin*dIII/*Acc*I fragment containing the Jκ1-Jκ2 region, the 0.4-kb AccI/AccI fragment containing the Jκ3-Jκ4 region, and the 1.6-kb *Acc*I/*Hin*dIII fragment containing the Jκ5 region.

DNA fragments were isolated from the plasmid by the freeze and squeeze method. In short, DNA digests were run on an NA agarose gel (Pharmacia) and the bands were cut out the gel and frozen. The frozen blocks were put between parafilm and the solution was squeezed out (usually with a glass plate), followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation [32]. Probes were made by labeling the isolated inserts with [α-^{32}P]dCTP (NEN research products, 3000 Ci/mmol) by multi-primed elongation [7].

Primers

The 17mer anti-sense Cκ oligonucleotide [3] and the sense L3-L7 Vκ oligonucleotides [14] were used as primers for cDNA synthesis

and amplification of the light-chain transcripts, the 17mer anti-sense IgG1 Cγ oligonucleotide [3] and the sense Vh5.1 and Vh5.2 [17] oligonucleotides as primers for the cDNA synthesis and amplification of the heavy-chain transcripts, respectively.

Cκ: AGATGGATACAGTTGGT
L3: AGTTCCGAGCTCGTGCTCACCCAGTCTCCA
L4: AGTTCCGAGCTCCAGATGACCCAGTCTCCA
L5: AGATGTGAGCTCGTGATGACCCAGACTCCA
L6: AGATGTGAGCTCGTCATGACCCAGTCTCCA
L7: AGTTCCGAGCTCGTGATGACACAGTCTCCA
Cγ: GGGGCCAGTGGATAGAC
Vh5.1: GAGGTGAAGCTGGTGGAG(A/T)C(A/T)GG
Vh5.2: CAGGTCCAGTTGCAGCAG(A/T)C(A/T)GG

The universal-sequence primers for pUC19 were obtained from Pharmacia. The 18mer primer used to sequence the Jh2 region in the genomic clones was complementary to the sequence TAAAACCTCTCTCTTCTA, located 9 base pairs (bp) downstream from the Jh2 gene segment. The 18mer primer used for sequencing in the Jκ5 region in the genomic clones was complementary to the sequence GACACAGGTTTTCATGTT located 35 bp downstream from the Jκ5 gene segment.

Reverse transcriptase/polymerase chain reaction (RT-PCR)

RNA was isolated from the E48 hybridoma cell line according to Gough [12]. cDNA was synthesized on 5 μg RNA by AMV reverse transcriptase primed by 5 pmol antisense Cκ and Cγ chain oligonucleotides respectively. PCR was performed in 50-μl reactions covered with paraffin oil using the following conditions: 25 pmol sense and antisense primers, 50 mM KCl, 10 mM TRIS-HCl pH 8.3, 1.5 mM $MgCl_2$, 200 μM dNTP and 1 U Taq polymerase (AmpliTaq, Cetus: Perkin Elmer Nederland B.V., Gouda, The Netherlands). 40 cycles of 1 min at 95° C, 2 min at 50° C and 3 min at 72° C were run in a BioMed PCR processor (Tecnolab, Alkmaar, The Netherlands). PCR fragments were treated with T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) according to the suppliers using 1 mM ATP and 200 μM dNTP in the reaction mix. The PCR fragments were isolated from NA agarose gels (Pharmacia), ligated into pTZ18 or pUC19 vectors and transformed into *E. coli* K12 DH5α. Clones containing PCR fragments were sequenced by the dideoxy-DNA chain-termination method using T7 DNA polymerase.

Southern blotting and hybridization

DNA was isolated from hybridoma cells according to Brakenhoff et al. (2). A sample containing 10–15 μg DNA was digested to completion with restriction enzymes, loaded on a 0.7% agarose gel and electrophoresed in TRIS-Acetate/EDTA buffer according to Sambrook et al. [32]. The DNA was denatured by soaking the gel in 0.4 M NaOH/0.6 M NaCl for 30 min, and blotted by capillary transfer to Genescreen-plus filters (NEN research products) in the same solution. After transfer the blot was neutralized in 1 M NH_4OAc/0.02 M NaOH for 5 min and washed in 2 × standard saline citrate (SSC). The filter was baked for 2 h at 80° C, prehybridized in 7% sodium dodecyl sulfate (SDS)/0.5 M sodium phosphate buffer/2 mM EDTA, pH 7.0 for 2 h at 65° C and, after addition of the denatured probe, hybridized at 65° C for 16 h. Filters were washed twice with 2 × SSC/0.2% SDS and twice with 0.2 × SSC/0.2% SDS at 65° C for 15 min, and the bands visualized by autoradiography with Kodak X AR-5 film (Kodak, Odijk, The Netherlands) using intensifying screens.

Molecular cloning of E48 light- and heavy-chain genes

The gene encoding the E48 heavy chain was cloned as follows: 100 μg genomic E48 hybridoma DNA was digested to completion with *Eco*RI and fractionated by gel electrophoresis on a 0.7% NA agarose gel (Pharmacia). DNA was recovered by the freeze and squeeze method and the fractions enriched for the 3-kb or 4-kb fragments were identified by Southern blotting and hybridization with the mJh probe. DNA from the enriched fractions was ligated into λ ZAPII vector (Stratagene, Westburg, Leusden, The Netherlands), and clones hybridizing to the mJh probe were plaque-purified, subcloned by in vivo excision, and sequenced in the VDJ, 5′ and 3′ end regions.

The 4-kb *Hin*dIII gene containing the E48 light-chain gene was cloned as follows: genomic E48 hybridoma DNA was partially digested by *Sau*3AI and fractionated by sucrose-gradient centrifugation [32]. The 20-kb fraction was ligated into λ EMBL3 vector arms (Promega), and the phage libary plated on *E. coli* MB406 (Promega), a strain known for its ability to propagate unstable sequences [42]. A total of 1.2×10^6 recombinants were screened with the 1.6-kb *Hin*dIII/*Acc*I fragment containing the Jκ5 gene segment as a probe, and 28 positive clones were rescreened with the 0.4-kb *Hin*dIII/*Acc*I fragment containing the Jκ3/Jκ4 gene segments as well as with the E48-light-chain-encoding cDNA clone. Three clones were positive with the Jκ5 and E48 cDNA but not with the Jκ3/Jκ4 probe, and these were plaque-purified. Restriction mapping on DNA isolated from these clones revealed that one clone contained the correct full length insert, one clone appeared to be recombined in the 3′ region, and the third clone missed one of the *Hin*dIII sites. The 4-kb *Hin*dIII fragment of the apparently correct clone was subcloned in pUC19. Restriction mapping and sequence analysis of the VJ region and the 3′ end region confirmed that the clone contained the correct insert.

Generation of chimeric-antibody-producing cell lines

The genomic fragments containing the genes encoding the variable regions of the light and heavy chains of mAb E48 were inserted into the heavy- and light-chain expression vectors as previously described [22]. The DNA constructs, previously digested with *Bam*HI, were transfected into the myeloma cell line 653 by electroporation as described earlier [22]. Transfectants were selected in 0.5 μg/ml mycophenolic acid, 2.5 μg/ml hypoxanthine and 50 μg/ml xanthine (Sigma, Bornem, Belgium). Clones were assayed for antibody production by a standard enzyme-linked immunosorbent assay (ELISA) using plates coated with anti-(human IgG) (Fc-fragment-specific) and goat anti-(human IgG (H + L) antibody conjugated to alkaline phosphatase (Jackson Immuno Research, West Grove, Pa., USA). cmAb was purified from the medium by protein-A–Sepharose chromatography (Pharmacia).

Immunohistochemistry, sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting

Immunohistochemistry on cells cytospun on glass microscope slides and frozen sections of squamous cell carcinoma xenografts was performed as described previously [26]. For Western blotting, cultured UM-SCC-14C and UM-SCC-22A cells were lysed in Laemmli sample buffer without 2-mercaptoethanol [19]. Lysates were boiled, centrifuged, and applied on a miniprotean II system (Bio-Rad, Veenendaal, The Netherlands) for sodium dodecyl sulfate/polyacrylamide gel electrophoresis performed as described by Laemmli, using a 12.5 slab gel [19]. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose (Schleicher & Schüll, Dassel, Germany) was performed by a mini-transblot electrophoretic cell (Bio-Rad), at 100 V for 1 h [32]. After transfer, the nitrocellulose

membrane was cut into strips and incubated at room temperature with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to block free binding sites on the membrane. The "blocked" nitrocellulose membrane strips were incubated for 1 h with either ^{131}I-labeled cmAb E48 or ^{125}I-labeled mmAb E48 (100,000 cpm/ml). Finally, nitrocellulose strips were washed 3 times for 10 min with PBS, dried, and autoradiographed by exposure to X-ray film for 1 day at $-70°$C.

Radioiodination and in vitro binding assays

Radioiodination of mAb and determination of the immunoreactive fraction in the immunoreactivity assay was performed as described earlier [8]. The immunoreactive fraction was always larger than 80%. Assessment of the affinity constant was performed essentially as described by Badger et al. [1]. Samples containing 5×10^6 UM-SCC-22A cells were fixed with 1% paraformaldehyde in PBS and incubated overnight in PBS/1% BSA at room temperature with 5000 cpm labeled mAb and a serial dilution of unlabeled mAb. The concentration of unlabeled mAb was chosen to be several times higher and several times lower than the concentration of the radiolabeled mAb as calculated from the specific activity. Cells were spun down, radioactivity in the pellet and supernatant was determined in a gamma counter (Wallac CompuGamma 1282: E. G. and G., Wallac, Nieuwegein, The Netherlands) and the percentage of bound and free radiolabeled mAb calculated. Data were graphically analyzed by Scatchard's analysis and the affinity constant determined. Affinity assays were performed in triplicate.

Biodistribution in tumor-bearing nude mice

In vivo biodistribution was studied in nude mice bearing human squamous cell carcinoma xenografts of the head and neck. The tumor xenograft line HNX-HN was established and maintained in female nude mice (Hsd, athymic nude *nu*, 25–32 g; Harlan/CPB Zeist, The Netherlands) as described earlier [8]. Five nude mice bearing nine xenografts were simultaneously injected with 9.1 μCi ^{131}I-labeled cmAb E48 (50 μg) and 2.3 μCi ^{125}I-labeled mmAb E48 (50 μg). At the time of injection the estimated xenograft volume, as determined by measuring the tumor in three dimensions with callipers [(length × width × height/2], was 509 ± 250 mm^3. Mice were bled, killed, and dissected 3 days after injection. Organs were immediately removed, placed in 5-ml plastic tubes and weighed. Samples were taken from blood, urine, tumor, liver, spleen, kidney, heart, stomach, ileum, colon, bladder, sternum, muscle, lung, skin and tongue. After weighing, radioactivity in the organs and tumor was counted in a dual-isotope gamma counter (Wallac CompuGamma 1282). Standards were included to correct for the contribution of both isotopes in the various window settings. The antibody uptake for both isotopes was calculated as the percentage of the injected dose per gram of tissue (%ID/g). All animal experiments were performed according to the principles of laboratory animal care (NIH publication 85-23, revised 1985) as well as the Dutch national law "Wet op de Dierproeven" (Stb 1985, 336).

Antibody-dependent cellular cytotoxicity

UM-SCC-22A cells served as target cells, and peripheral blood mononuclear cells (PBMC) from healthy volunteers as effector cells in ADCC assays. PBMC were isolated from whole blood by separation on Ficoll-Paque gradients (Pharmacia). Cells collected from the interface were washed twice in Hanks' balanced salt solution (HBSS, Gibco Life Technologies) by centrifugation, and incubated overnight in DMEM/5% FCS in 5% CO_2 at 37° C.

Target cells were trypsinized by 0.05% trypsin/0.02% EDTA in PBS (Gibco Life Technologies), washed with tissue-culture medium, and resuspended in tissue-culture medium at a concentration of 10^5 cells/ml. To 1 ml suspended cells 50 μCi sodium [^{51}Cr]chromate (332 mCi/mg) was added, and 5×10^3 cells (50 μl) were plated in the wells of 96-well U-bottomed plates (Greiner N. V./S. A., Westmalle, Belgium). After incubation of the plates for 16 h in 5% CO_2 at 37° C, the cells were washed three times with DMEM/5% FCS. After washing, effector cells were added at an effector: target cell (E:T) ratio of 50:1 or different E:T ratios ranging from 6.25:1 to 100:1, and mAb were added to a final concentration ranging from 0.001 μg/ml to 10 μg/ml or 10 μg/ml respectively. Plates were centrifuged at 65 g for 30 s and incubated in 5% CO_2 at 37° C for 5 h. Medium was harvested from each well and radioactivity counted in a gamma counter (Wallac CompuGamma 1282). All assays were performed in triplicate. Maximal isotope release was determined by incubation of target cells in the presence of 5% Triton X-100. Natural killer (NK) cell release (antibody-independent lysis) was measured by incubation of target/effector cells in the presence of a murine IgG1 control anti-myosin mAb (Centocor Inc., Malvern Pa., USA), which does not bind to the target cells. The percentage specific lysis was calculated as [(experimental release − background release)/(Triton release − background release)] × 100. Background release is defined as the release in the absence of specific antibody (= spontaneous release + NK release). Standard deviations were typically 0–8%. Spontaneous chromium release never exceeded 20%, while antibody independent killing was less than 10%.

Results

Characterization and cloning of the E48 heavy-chain gene

Immunoglobulin transcripts of the E48 heavy-chain gene were amplified by RT-PCR using the Vh5.1, Vh5.2 and IgG1 Cγ primers as described by Kavaler et al. [17] and Caton et al. [3] respectively. Amplified fragments were isolated from NA agarose gels, cloned into pTZ18 or pUC19 vectors and partially sequenced in order to identify the rearranged genes that are expressed (see below). Sequence analysis of the cDNA clones revealed a rearrangement to the Jh2 gene segment (data not shown).

Southern blot analysis of *Eco*RI-digested genomic DNA of the E48 hybridoma, hybridized with the 2-kb *Eco*RI/*Bam*HI fragment containing the mouse Jh3 and Jh4 gene segments as a probe, revealed that the alleles of the parental lymphocyte had been rearranged to 3-kb and 4-kb *Eco*RI fragments, respectively (Fig. 1A). These fragments were cloned as described in Materials and methods. The 4-kb fragment contained the E48 heavy-chain gene, and the sequence in the VDJ region was identical to the amplified cDNA sequence with a rearrangement to the Jh2 gene segment. The 3-kb fragment contained an illegitimate rearrangement in the Jh2 region with considerable deletions of germline DNA (data not shown).

Characterization and cloning of the E48 light-chain gene

Immunoglobulin transcripts of the E48 light-chain gene were amplified by RT-PCR using the Vκ L3-L7

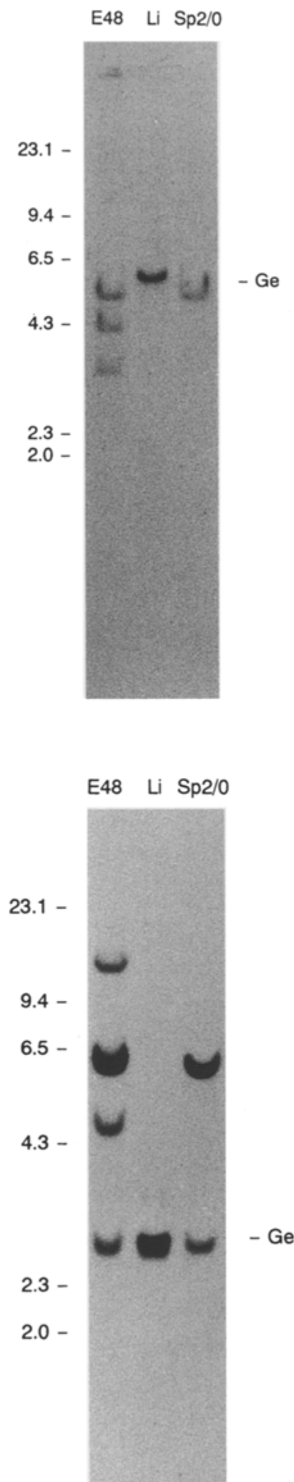

Fig. 1A, B DNA from BALB/c liver (*Li*), myeloma cell line Sp2/0 and hybridoma cell line E48 was cut with *Eco*RI (**A**) or *Hin*dIII (**B**), run on an agarose gel, Southern-blotted, hybridized with the mJh probe (Jh3–4 region, **A**) or mJk probe (Jκ1–5 region, **B**) and autoradiographed for 64 h. The germline fragment is indicated with *Ge*. The fragment lengths are indicated on the left (kb). Note the additional rearranged fragments in E48 hybridoma DNA: 3-kb and 4-kb *Eco*RI fragments in **A**, and 4-kb and 15-kb *Hin*dIII fragments in **B**, either of which could contain the gene encoding the variable domain of the E48 heavy chain or light chain respectively

and Cκ primers as described by Huse et al. [14] and Caton et al. [3] respectively. Amplified fragments were isolated from NA agarose gels, cloned into pTZ18 or pUC19 vectors and partially sequenced in order to identify the rearranged genes that are expressed (see below). The sequence of the light-chain transcripts indicated a rearrangement to the Jκ5 gene segment.

Southern blot analysis of *Hin*dIII-digested genomic DNA of the E48 hybridoma, hybridized with the 1.6-kb *Hin*dIII/*Acc*I fragment containing the germline Jκ5 fragment as a probe, indicated that in the light-chain locus the germline alleles of the parental lymphocyte had been rearranged to 4-kb and 15-kb *Hin*dIII fragments respectively (Fig. 1B). Since the sequencing data of the RT-PCR fragments indicated that the E48 light-chain gene contained a rearrangement to Jκ5, we subdivided the 2.8-kb *Hin*dIII fragment containing the germline Jκ region by *Hin*dIII/*Acc*I double digestion into three smaller fragments containing the Jκ1/Jκ2, Jκ3/Jκ4 and Jκ5 gene segments respectively. Southern blots of E48 genomic DNA hybridized with this set of probes indicated that the 4-kb *Hind*III fragment contained a rearrangement to Jκ5. No signal could be detected after hybridization with the Jκ1/Jκ2 or Jκ3/Jκ4 probes. The 15-kb fragment, however, hybridized with all probes and thus contained an illegitimate rearrangement or a rearrangement to Jκ1 or Jκ2 (Fig. 2). The 4-kb fragment was cloned as described in Materials and methods. The sequence was identical to the germline sequences at the 3′ end region, and identical to the E48 cDNA sequence in the VJ region.

Production and binding specificity of chimeric antibody

The genomic clones were inserted in the expression vectors p412HG1apgpt and p412HuKapgpt in front of the human κ and IgG1 constant domains respectively, and transfected into myeloma cells as described in Materials and methods. Highly producing cell lines were selected by limiting dilution. On the laboratory scale the production of chimeric antibody was approximately 5–10 μg/ml in tissue-culture flasks and 100–200 μg/ml in a Cell-Pharm hollow-fiber minibioreactor 100 (Unisyn Fibertec Inc., San Diego Calif., USA). In large-scale fermentors (50 l) the yield of antibody using this expression system is usually between 100 μg/ml and 500 μg/ml [22].

Binding specificity of the chimeric antibody was checked by immunocytochemical staining on cytospins of various squamous cell carcinoma cell lines, by immunohistochemical staining on frozen sections of various SCC xenografts, and by Western blotting. Western blots from antigen-negative UM-SCC-14C lysates and antigen-positive UM-SCC-22A lysates were incubated with radioiodinated cmAb or mmAb followed by autoradiography (Fig. 3). Both antibodies recognize

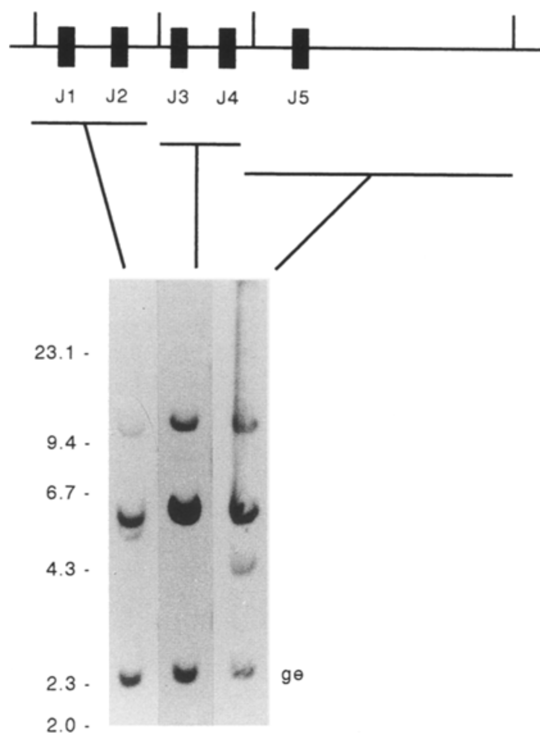

Fig. 2 DNA from hybridoma cell line E48 was cut with *Hin*dIII and hybridized with the Jκ1–2, Jκ3–4 and Jκ5 probe, respectively. A schematic physical map of the mouse Jκ1–5 (J1–J5) region is indicated on top. Note the presence of the 4-kb fragment only when the blots are hybridized with the Jκ5 region as a probe, indicating that the gene, rearranged to the Jκ5 gene segment, and encoding the variable domain of the E48 light chain, is located in the 4-kb *Hin*dIII fragment.

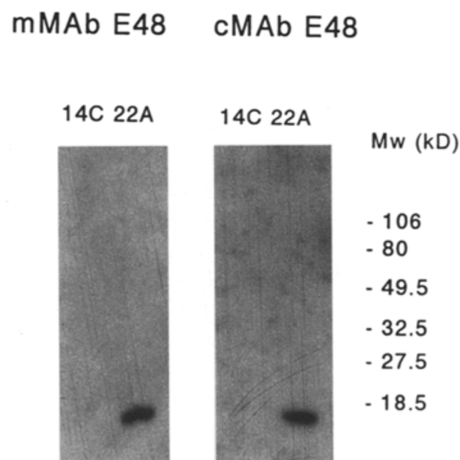

Fig. 3 Autoradiograph of the radio-immunoblot assay with mmAb E48 and cmAB E48 on cell lysates of an E48-antigen-negative cell line (UM-SCC-14C) and an E48-antigen-positive cell line (UM-SCC-22A). The molecular masses of protein standards run in parallel are indicated on the right

the same E48 antigen of approximately 15–20 kDa, and do not cross-react with other proteins [26]. Binding affinity was measured by Scatchard's analysis (data not shown). The affinity constant of the cmAb appeared to be $1.6 \times 10^{10}\,M^{-1}$ and that of the mmAb $0.9 \times 10^{10}\,M^{-1}$.

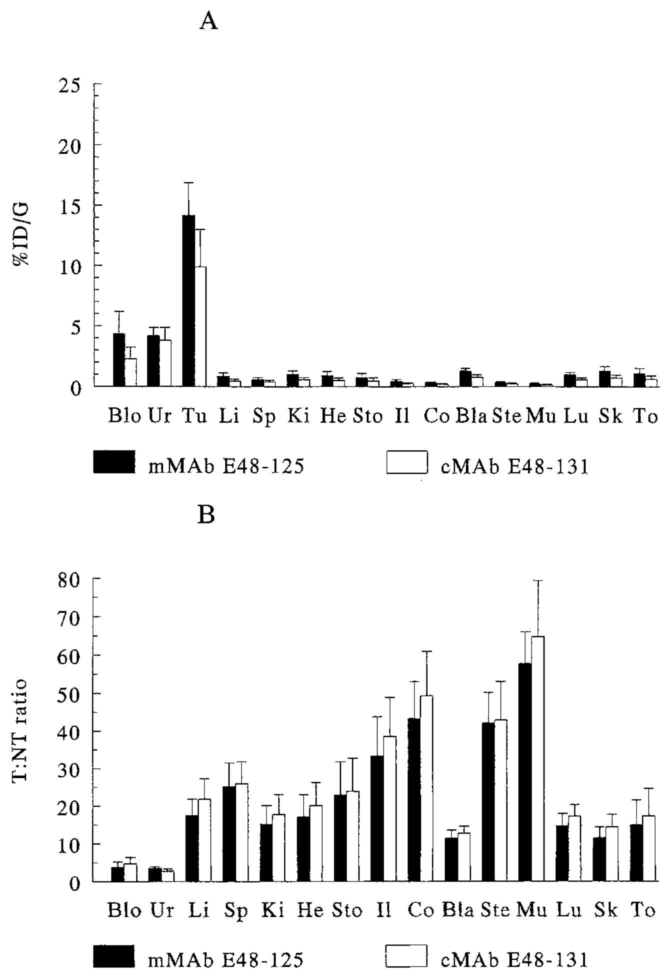

Fig. 4A, B Tissue uptake (**A**) and tumor to non-tumor values (**B**) of 9.1 μCi (50 μg) ^{131}I-labeled cmAb E48 (*open bars*) and 2.3 μCi (50 μg) ^{125}I-labeled mmAb E48 (*black bars*) in athymic mice bearing HNX-HN xenografts. At 3 days following i.v. injection, tissues were dissected and counted, and the percentage of the injected dose per gram (*%ID/G*) was calculated. Number of mice: 5, number of tumors: 9. *Blo* blood, *Ur* urine, *Tu* tumor, *Li* liver, *Sp* spleen, *Ki* kidney, *He* heart, *Sto* stomach, *Il* ileum, *Co* colon, *Bla* bladder, *Ste* sternum, *Mu* muscle, *Lu* lung, *Sk* skin, *To* tongue

In vivo biodistribution of cmAb E48 and mmAb E48

Although the specificity and affinity of the chimeric antibody were demonstrated by several lines of evidence, we could not rule out the possibility that the newly introduced human constant domain and its specific glycosylation would alter the pharmacokinetic properties of the antibody in vivo. Therefore we measured the biodistribution of 9.1 μCi ^{131}I-labeled cmAb E48 (50 μg) and 2.3 μCi ^{125}I-labeled mmAb E48 (50 μg) co-injected into xenograft-carrying nude mice 3 days after infection (Fig. 4A). For ^{131}I-labeled cmAb E48 the mean uptake was 2.3% Ig blood, which is 46% less

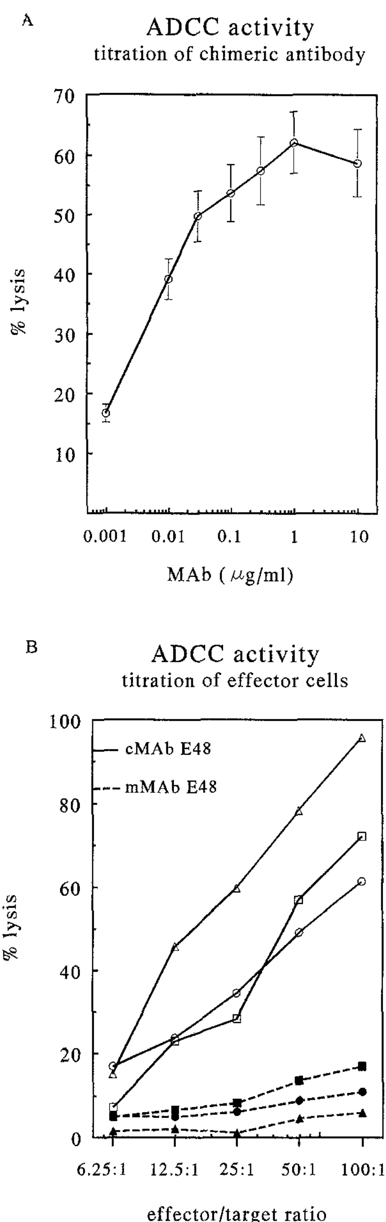

Fig. 5A, B Antibody-dependent cellular cytotoxicity (*ADCC*) activity of chimeric and murine mAb E48 in 5-h ^{51}Cr release assays. **A** Titration curve of cmAb E48. ADCC activity of cmAb E48 was determined using different concentrations of antibody (indicated on the *x*-axis), at an effector/target ratio of 50 : 1. The percentage lysis is given on the *y*-axis. **B** Comparison of ADCC activity of mmAb and cmAb E48. UM-SCC-22A target cells and human PBMC effector cells of six healthy volunteers were cultured in the presence of 10 μg/ml mAb at effector/target ratios of 6.25 : 1, 12.5 : 1, 25 : 1, 50 : 1 and 100 : 1 respectively. —— cmAb-E48-dependent release; - - - mmAb-E48-dependent release

than for mmAb E48, for which this value was 4.3% (Fig. 4A). Uptake in tumor tissue of cmAb E48 and mmAb E48 was 9.9%ID/g and 14.2%ID/g respectively, a 30% lower value for cmAb E48 than for mmAb E48. Uptake in all other tissues was less than 0.8%ID/g for cmAb and 1.3%ID/g for mmAb E48. For cmAb E48, tumor to non-tumor ratios 3 days after infection were 4.6 for blood, 11.7 for bladder, 12.8 for skin, and higher than 16 for any other tissue tested. These tumor to non-tumor values were essentially the same as those found for mmAb E48 (Fig. 4B).

ADCC activity of cmAb E48 and mmAb E48

The improved biological activity of chimeric mAb in antibody-dependent cellular cytotoxicity was measured in in vitro ADCC assays. Firstly, ADCC activity of chimeric mAb was tested in a 5-h ^{51}Cr-release assay using UM-SCC-22A as target cells, PBMC of a healthy volunteer as effector cells and antibody in a concentration range from 0.001 μg/ml to 10 μg/ml. As shown in Fig. 5A, the ADCC activity appeared to be dependent on antibody concentration, reaching its maximum level at 1 μg/ml (Fig. 5A). Secondly, the ADCC activity of chimeric (human IgG1) and murine mAb (murine IgG1) E48 was compared at the saturating mAb concentration of 10 μg/ml and using effector cells isolated from the blood of six healthy volunteers. When cmAb E48 was included in this assay at an effector/target ratio of 100 : 1, specific lysis percentages of 62%, 72% and 96% were found, while for mmAb E48 these values were 6%, 11%, and 17% respectively (Fig. 5B).

Discussion

The two main rationales for the chimerization of murine mAb to mouse/human mAb for tumor targeting are: (a) to decrease the immunogenicity of the mAb, and (b) to increase the potential for mediating tumor cell lysis by ADCC. Various investigators have already described the chimerization of antibodies, and various expression systems have been published. One of the expression systems that has proven its value in the production of large quantities of antibody in large-fermentor-scale production, makes use of the gene sequences encoding the variable domains of the light and heavy chains, respectively [22]. We therefore set out to achieve the molecular cloning of the genes encoding the E48 heavy- as well as light-chain variable domains. The determination of the restriction fragments containing these genes, however, was hindered by the observation that the light- as well as the heavy-chain genes had been rearranged on both chromosomal loci. We therefore determined the rearrangement pattern by PCR amplification and subsequent sequencing of the cDNA of the E48 genes being expressed. The elucidation of this rearrangement pattern then facilitated the identification of the *Hin*dIII fragment containing the E48 light-chain gene, enabled the selection of the clones containing Jκ5 rearranged V genes from the constructed genomic library by differential hybridization, and simplified the evaluation of the various isolated genomic

clones. The molecular cloning of the E48 heavy-chain gene was straightforward, but the cloning of the 4-kb *Hin*dIII fragment containing the E48 light-chain gene was much more difficult. A first attempt to clone the *Hin*dIII fragment by the construction of a sublibrary from fractionated *Hin*dIII-digested genomic DNA in a λ Charon 27 *Hin*dIII insertion vector did not result in the isolation of a single specific clone, most likely because of instability of the sequences in *E. coli*. Therefore a genomic library was prepared in an *E. coli* strain especially selected for the cloning of unstable sequences [42]. Even in this particular strain recombined clones were found, and only one of the three clones finally selected was shown to contain the correct insert.

At the time when the chimerization project of mAb E48 was started, only limited data on the immunogenicity of mAb E48 were available. A clinical radioimmunoscintigraphy study in head and neck cancer patients who received 1–2 mg radiolabeled mAb E48 $F(ab')_2$ had just been finished [6]. Out of 16 patients, 3 (19%) developed a HAMA response as assessed with a mmAb-E48-based HAMA assay (manuscript in preparation) essentially according to a method described by Massagur et al. [23]. This percentage was low in comparison to the HAMA incidence of 50%–75% as generally found in patients with other solid tumors receiving mAb [18]. On the basis of these data it was tempting to state that the immunogenicity of mAb E48 $F(ab')_2$ is low. Other explanations were related to the immunosuppressed state as frequently observed in head and neck cancer patients [40] and the relatively low dose of mAb used in these studies. Recently, Seybold et al. [34] evaluated a long-term HAMA follow-up after immunoscintigraphy using anti-granulocyte and antitumor mAb in 230 patients. After a first injection of 0.15–1 mg the HAMA incidence was typically about 9%. The incidence increased when more mAb protein was applied, especially after repeated injections. A similar observation has been described by Courtenay-Luck et al. [5].

At our institute, the infrastructure is being organized for a clinical radioimmunotherapy trial in which ^{186}Re-labeled cmAb E48 will be evaluated for the adjuvant treatment of patients with a high risk for developing local recurrence and distant metastases [37]. To this end an efficient and reproducible technical protocol for aseptic production of stable ^{186}Re-mAb conjugates was recently developed [39]. Using this unique solid-phase synthesis with the MAG3 chelate, conjugates with an isotope:mAb ratio of at least 8:1 can be generated having full binding capacity and unaltered biodistribution in tumor-bearing nude mice. This high ratio enables the preparation of ^{186}Re conjugates for clinical purposes. In nude mice bearing small head and neck cancer xenografts (75 mm^3) 100% cures could be obtained using these ^{186}Re-labeled mmAb conjugates [11].

When using cmAb E48 in adjuvant clinical radioimmunotherapy studies, however, it can be anticipated that ADCC activity may be supportive to irradiation, especially in eradicating single disseminated cells or small cell aggregates. In the present study we show that cmAb E48 is capable of lysing HNSCC targets in ADCC assays in vitro, whereas the mmAb appeared to be almost inactive. The enhanced ADCC activity observed after replacement of the murine γ1 constant region of mAb E48 with the human γ1 constant region is consistent with findings of others who showed that IgG1 is the most effective human isotype in mediating ADCC with human effector cells whereas murine IgG1 is relatively ineffective [35, 36]. With respect to the use of cmAb E48 in adjuvant ^{186}Re radioimmunotherapy, it is of note that the ADCC-mediating capacity of cmAb E48 did not become impaired upon coupling of eight MAG3 chelate groups (manuscript in preparation). In these experiments the mAb concentration was titrated down to 0.1 μg/ml.

In other clinical studies we will focus on the use of unlabeled cmAb E48 for adjuvant therapy of head and neck cancer. The feasibility of this approach was recently demonstrated in a randomized trial in which mmAb 17-1A (IgG2a) was used in adjuvant therapy of resected Dukes' C colorectal carcinoma [31]. After a mean follow-up of 5 years, antibody treatment appeared to have reduced the overall death rate by 30%. Treatment with mAb 17-1A prolonged distant relapse while no effect was seen on local relapse. Antitumor effects with mAb 17-1A were also observed in tumor-bearing nude mice and in these studies evidence was found for an effector-cell-dependent cytotoxic mechanism [13]. We think that these data, in combination with the ADCC-mediating potential of cmAb E48, as observed in this study, justify the clinical evaluation of cmAb E48 for its effectiveness in adjuvant therapy of head and neck cancer.

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