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Original Paper

Anti-idiotypic Response to Antigrowth Factor Receptor Monoclonal Antibodies

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The immunogenicity of the idiotypic portions of two antigrowth factor receptor monoclonal antibodies (MAbs) was studied. Immunisation of allogeneic but not syngeneic mice with antihuman epidermal growth factor receptor (EGF-R) MAb MINT5 or anti-HER-2/neu MGR6 MAb elicited a detectable titre of circulating antibodies, particularly when the MAb was coupled with the keyhole limpet haemocyanin and administered together with Freund's adjuvant. The anti-Ab1 response to MAb MINT5 was slightly delayed as compared with the response obtained with MAb MGR6 and was mainly directed to the variable regions. In both cases, all anti-Ab1-positive sera specifically competed with the binding of homologous radiolabelled Ab1 to the relevant EGF-R+ or HER-2/neu+ target cells. Fusion of splenocytes from MINT5-immunised animals failed to produce MAb, whereas cell fusion was successful in generating a paratope-related MAb in the case of MGR6. The anti-MGR6 MAb-produced IdM6.4 inhibited the binding of MAb MGR6 on breast carcinoma cells, suggesting that it recognises an idiotope in or near the antigen combining site, and can be considered useful in the identification and purification of the Ab1 or its derivatives. We analysed whether a possible recognition of murine EGF-R by MAb MINT5 or a mimicry of EGF by the MAb idiotype prevented or delayed the development of an idiotypic cascade in mice. MINT5 inhibited human and murine EGF binding to the human EGF-R, whereas the anti-Ab1 response competed with MINT5 but not with murine EGF binding to A431 human epidermoid carcinoma cells. Moreover, MINT5 did not recognise the murine EGF-R. In a phase I clinical study, no detectable levels of human antimouse antibody response were observed in 5 of the 6 treated cancer patients. The ability of MAb MINT5 to block human EGF-R function, together with its low immunogenicity in patients, raise the possibility of its application in carcinoma immunotherapy.

Key words: growth factor receptor, HER-2/neu, EGF-R, idiotype, monoclonal antibodies
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INTRODUCTION

THE IDIOTYPIC network theory [1, 2] is based on the immunogenicity of the variable regions of immunoglobulin molecules (Ab1) which stimulate the immune system to generate anti-Id Abs (Ab2), some of which mimic antigenic epitopes ("internal image" anti-Id). The concept of idiotypy has stimulated a great deal of research and has led to a novel method for generating antibodies to pathogens and cell receptors. Analysis of the idiotypic network has proven to be useful in isolating ligands and/or receptors, and anti-Id monoclonal antibodies (MAbs) have been used as surrogate antigens in active specific immunotherapy (for review see [3, 4]). Numerous Ab2 MAbs that mimic tumour-associated antigens, such as the high-molecular-weight melanoma-associated antigen [5], carcinoembryonic antigen [6] and ovarian carcinoma gp38 antigen [7, 8], have been produced and characterised. The ability of Ab2 to induce an Ab1-like response (Ab3) has been investigated in animal models [9], whereas in humans the Ab3 effect on tumour growth has been evaluated following Ab2 or Ab1 treatment [10–15].

In the present report, we describe our analysis of the anti-Ab1 response induced against MAbs MINT5 and MGR6. These MAbs recognise the growth factor receptors EGF-R and HER-2/neu, respectively, which are expressed at low

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levels in normal cells, and are overexpressed in several human carcinomas [16]. Although neither EGF-R nor HER-2/neu are tumour-specific antigens, their overexpression is correlated with poor prognosis [17–20] and can be considered a suitable target for therapy.

The biological relevance of the interaction of these two MAbs with their respective growth factor receptors is suggested by the observation that MINT5 cross-competes with the natural ligand EGF and downmodulates EGF-R [21], while MGR6 induces downmodulation and tyrosine phosphorylation of HER-2/neu [22, 23]. Moreover, MINT5 in preclinical *in vivo* models exhibited a therapeutic efficacy against human carcinomas which is based on its interference with the EGF-R-TGFα autocrine/paracrine loop [21].

In a murine model, we found that induction of an anti-Ab1 response against the two MAbs requires an allogeneic host, adjuvant and carrier. The anti-MGR6 response was earlier, and one paratope-related MAb was generated from cell fusion of splenocytes from MGR6-immunised animals. The anti-MINT5 response appeared to be directed mainly to the variable regions of the MAb but not against the natural ligand. In a clinical setting, preliminary results indicate the very low immunogenicity of MAb MINT5.

MATERIALS AND METHODS

Mice

Female Balb/c, C57BL/6 and nu/nu CD1 mice (4–8 weeks old) were purchased from Charles River Laboratories (Calco, Como, Italy), and housed and maintained under pathogen-free conditions.

Cell lines

The human cell lines A431 (vulvar carcinoma) and SKBr3 (breast carcinoma) were obtained from ATCC (Rockville, Maryland, U.S.A.); the cell lines MeWo (melanoma) and IGROV1 (ovary carcinoma) were kindly provided by the late Dr J. Fogh (Memorial Sloan Kettering Cancer Center, New York, U.S.A.) and by Dr Bénard (Institute Gustave Roussy, Villejuif, France), respectively. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% glutamine, penicillin (100 μg/ml) and streptomycin (100 μg/ml). The murine cell line 3T3-Swiss (ATCC) was grown in DMEM (Boehringer-Mannheim, Mannheim, Germany) supplemented with 10% calf serum (Colorado Serum Company, Denver, Colorado, U.S.A.), 1% glutamine and 0.1% gentamycin.

Antibodies

MAbs MINT5 (IgG1) and MGR6 (IgG2a) were developed and characterised as previously described [21–23]. Antihepatitis virus MAb 2AC6 (IgG1) and antidoxorubicin MAb MAD11 (IgG2a) were used as unrelated controls and were isotype-matched with MINT5 and MGR6. Anti-ovarian carcinoma MAb MOV18 (IgG1) [24] was used to evaluate the anti-idiotypic response of patients' sera in an idiotype inhibition assay (see below). All MAbs were of Balb/c origin. MAbs were purified from mouse ascites by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) and were radio-iodinated with $Na^{125}I$ (Amersham, Little Chalfont, U.K.) by the lactoperoxidase method [25] to a specific activity of 5–10 μCi/μg protein. MINT5 was biotinylated using a biotinylation kit (Amersham), following the manufacturer's instructions.

MAb coupling

Purified MAb was linked to mouse red blood cells (MRBC) as previously described [8]. Briefly, MAb (5 mg/ml in 0.15 M NaCl) was incubated with MRBC (1:1) for 1 h at room temperature in the presence of a 10-fold volume of 0.2 mM $CrCl_3.6H_2O$, and the mixture was washed three times with 0.15 M NaCl. The immunogen was freshly prepared before each immunisation and assessed for activity by direct immunofluorescence and by a rosette-forming assay, in which 10^6 relevant tumour cells were incubated with 10^7 MRBC-MAb or control MRBC for 1 h at 4°C and examined by light microscopy. The immunogens showed positive binding to the relevant tumour before each injection.

MAbs were coupled to keyhole limpet haemocyanin (KLH) (Sigma, St Louis, Missouri) essentially as previously described [26]. Equal volumes of MAb (6 mg/ml in 0.15 M NaCl) and KLH (6 mg/ml in PBS) were incubated with 0.25% glutaraldehyde for 30 min at room temperature. The reaction was stopped by addition of 2 M glycine to the mixture.

Immunisation protocols

Table 1 summarises the immunisation protocols in C57BL/6 mice. Time intervals between injections were: protocol I, 10-days; protocol II, 14-days; protocol III, 7 days. Complete Freund's adjuvant was used for the first injection and incomplete Freund's was used for subsequent injections. In protocols I and II, control mice were injected with MRBC, whereas in protocol III, control animals received 0.15 M NaCl. Individual mice were bled at different time intervals, and serum was stored frozen.

Analysis of anti-Ab1 response

Ab1 cross-linking assay was performed essentially as previously described [8]. This assay is based on the binding of one site of the soluble anti-Ab1 antibody (present in excess) to the coated Ab1 used for immunisation, so that unoccupied sites are available to bind subsequently added, soluble radiolabelled Ab1. Briefly, 2-fold serial dilutions starting from 1:20 of the anti-Ab1 sera were incubated in microtitre plates coated with MAb (1 μg/well) and tested for their ability to create a bridge between coated MAb and relevant ^{125}I-labelled MAb (10^5 cpm/50 μl).

Anti-carrier response was analysed in anti-Ab1 sera incubated on plates coated with KLH in PBS (3 μg/well). ^{125}I-labelled anti-mouse Ig antibodies were used as the detecting reagent (Amersham).

Washing, treatment with 2N NaOH, and evaluation of bound radioactivity were performed as previously described [8] in all assays.

Idiotype inhibition assay

Inhibition of ^{125}I-MINT5 or murine ^{125}I-EGF (Amersham) binding to A431 cells, of ^{125}I-MGR6 binding to SKBr3 cells, and of ^{125}I-MOV18 binding to IGROV1 cells was assayed by incubating serially diluted inhibitors with equal volumes of ^{125}I-labelled MAb or murine ^{125}I-EGF (input 1.3 nM) for 1 h at room temperature, incubating the mixture with the relevant cells for 45 min at 37°C, and calculating the percentage inhibition as follows:

$$\% \text{ inhibition} = (1 - Cs/Cm) \times 100,$$

where Cs is the average cpm in the presence of inhibitor and Cm is the average cpm without inhibitor.

Production of anti-Ab1 MAb

Spleen cells of C57BL/6 mice immunised according to protocols I and III were fused with NSO myeloma cells as described [27]; hybrids grown in HAT medium were screened by idiotype cross-linking assay. After two subclonings, hybrids secreting MAb were cultured *in vitro* and the MAb isotype was determined by indirect immunofluorescence using a FACScan (Becton-Dickinson, Mountain View, California, U.S.A.). Hybridoma cells were grown either *in vivo* in pristane-primed nu/nu CD1 mice or *in vitro* in standard cell culture conditions. MAbs were purified from mouse ascites and/or cell culture supernatant by affinity chromatography using Hi Trap^{TM} protein G (Pharmacia).

EGF-binding competition assay

A431 and 3T3-Swiss cells in 50 μl of culture medium supplemented with dialysed FCS and containing murine ^{125}I-EGF were incubated in the presence or absence of either cold recombinant human EGF (Amersham) or MAb MINT5 for 3 h at 0°C. After three washes, cells were assayed directly for radioactivity in a gamma counter.

Immunohistochemical assay

nu/nu CD1 mice were injected subcutaneously (s.c.) with 2.5×10^6 A431 cells. Tumours were recovered at necropsy when they reached a size of about 0.9×0.8 mm, and were preserved in liquid nitrogen. Cryostat preparations of murine epidermis and s.c. A431 solid tumours were fixed in cold acetone for 10 min and incubated with biotinylated-murine EGF (Boehringer-Mannheim) (20 μg/ml) or biotinylated-MINT5 (27 μg/ml) in Hanks' solution for 1 h at 37°C. Sections were then stained with peroxidase–biotin–streptavidin complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, California, U.S.A.) and 0.03% 3,3'-diaminobenzidine was used to detect the peroxidase activity.

HAMA response in cancer patients

Blood samples were collected from patients entered in phase I–II studies ([28] for MOV18 MAb and unpublished data for MINT5). Pretreatment sera and samples collected 15–20 and 30–40 days after a single injection of murine MAb (dose range 1–10 mg) were analysed for development of human antimouse antibody (HAMA) response by an ELISA-HAMA kit (Medac, Hamburg, Germany).

RESULTS

Development of the anti-Ab1 response

Immunisation with the two MAbs was initially conducted in the syngeneic Balb/c strain following different procedures. Ab1 cross-linking and idiotype-inhibition assays of mouse sera revealed no indication of an anti-Ab1 response, independent of the site and number of injections or the use of adjuvants (data not shown). Thus, allogeneic C57BL/6 mice were immunised with the MRBC-MAb complex in the presence of Freund's or Titer Max adjuvant (protocols I and II in Table 1). Two additional groups received, respectively, KLH-MINT5 and KLH-MGR6 complex with Freund's adjuvant (protocol III). The anti-Ab1 response of the allogeneic mice developed differently according to the MAb and the protocol used. As shown in Figure 1, the response was greatly enhanced by the use of the KLH carrier and the anti-MGR6 response (Figure 1a) became evident earlier than that against MINT5 (Figure 1b). The minimum number of injections needed to immunise C57BL/6 mice against MGR6 and MINT5, respectively, was four versus five with protocol I, and two versus three with protocol III. At the end of the immunisation protocols, the anti-Ab1 serum mean titres were higher in mice immunised with MINT5 (1:170 versus 1:50 for protocol I and 1:420 versus 1:220 for protocol III). The use of Titer Max adjuvant was successful only in MGR6 immunisations, inducing detectable anti-Ab1 response (mean titre 1:90) after a total injection of 80–150 μg of MRBC–MGR6 complex (five times less than the total amount inoculated with Freund's adjuvant).

Table 1. Immunisation protocols for anti-Ab1 response

	Protocol		
	I	II	III
Immunogen			
Complex	MRBC-MAb	MRBC-MAb	KLH-MAb
μg/injection	80–150*	40–75*	100
Adjuvant	FA	TM	FA
Injection			
Number	5	2	5
Site	i.p.	s.c.	i.p.
No. of positive sera†			
MGR6	4/4	2/3	4/4
MINT5	2/3	0/4	4/4

*The yield of MAb coupled to MRBC ranged from 30 to 60%. †Sera were considered positive in Ab1 cross-linking assay when binding at 1:20 dilution was at least 3-fold more than that of control sera. FA, Freund's adjuvant; TM, Titer Max adjuvant; i.p., intraperitoneally; s.c., subcutaneously.

Immune response in protocol III mice

The anti-KLH response was found to increase similarly during the course of immunisation with either MAb MGR6 or MINT5 and after two immunisations the mean titres were higher than 1:2560. The unrelated MAb 2AC6 and MAD11 were included at each timepoint to determine the presence of anti-isotypic and/or anti-allotypic components in the immune sera (Figure 2). Only mice treated with KLH-MGR6 showed an anti-allotypic response toward MAb 2AC6 and an anti-isotypic plus anti-allotypic response toward MAb MAD11. At the end of the immunisation, the serum mean titre of these responses were 1:35 and 1:120, respectively.

Specificity of the anti-Ab1 response

Binding of ^{125}I-MGR6 and ^{125}I-MINT5 to SKBr3 and A431 cells, respectively, was measured in the presence of different concentrations of immune sera obtained at the end of the immunisations using protocols I and III. All anti-MGR6-positive sera (Figure 3a,b) competed with the radiolabelled Ab1, although the level of competition in mice immunised with MRBC-MGR6 (Figure 3a) was more heterogeneous than in KLH-MGR6-immunised mice. For each protocol, the anti-MINT5 serum that gave the greatest inhibitory effect in homologous combination was used as control. All anti-MINT5-positive sera (Figure 3c,d) efficiently competed with Ab1, showing a specificity as high as that observed with the anti-MGR6 immune sera. For each protocol, the anti-MGR6 serum that gave the highest inhibitory effect in homologous combination was used as control. No inhibition

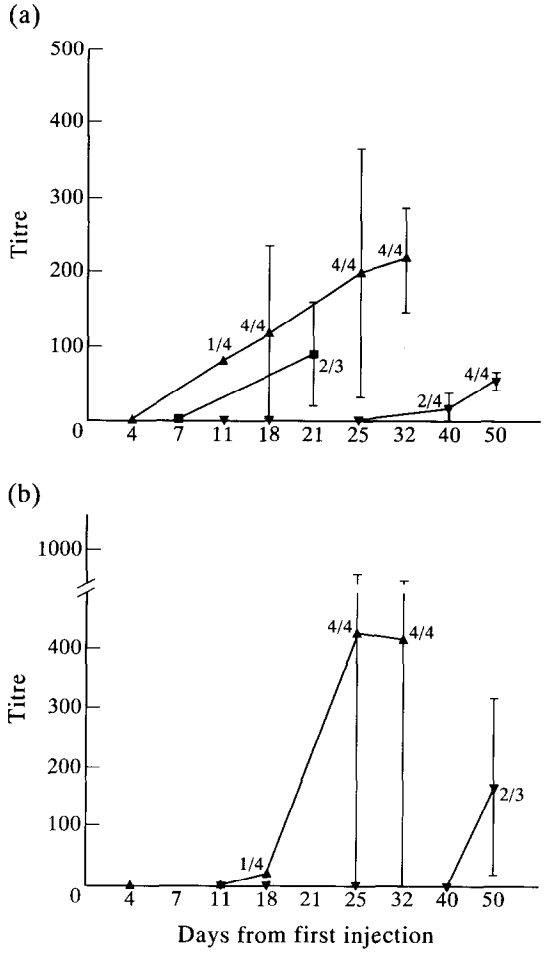

Figure 1. Time course of the anti-Ab1 response to MGR6 MAb (a) and MINT5 (b) in C57BL/6 mice treated with three different immunisation protocols. The response, as measured by the Ab1 cross-linking assay, is reported as the number of positive sera at each timepoint and the mean serum titre of positive sera. Titre is defined as the highest serum dilution at which binding is 10-fold higher than that of control sera. Sera of mice immunised with: MRBC-MAb + FA (▼), MRBC-MAb + TM (■), KLH-MAb + FA (▲). Error bars, ± S.D.

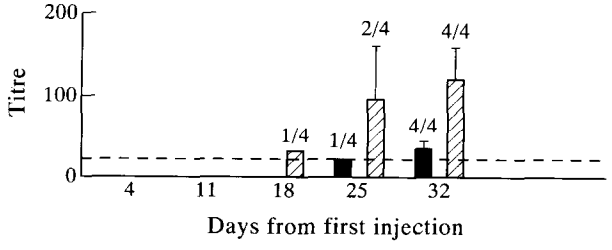

Figure 2. Time course of the immune response in protocol III mice. Anti-isotypic and -allotypic responses tested in the following Ab1 cross-linking assays: coated MAb MAD11 and 2AC6, respectively, for tracer ^{125}I-MGR6; coated MAb 2AC6 and MAD11, for tracer ^{125}I-MINT5. The response is reported as in Figure 1. ▨, anti-allotypic response against MGR6; ■, anti-isotypic plus anti-allotypic response against MGR6. Anti-allotypic response and anti-isotypic plus anti-allotypic response against MINT5 are below titre = 20 (dotted line). Error bars, S.D.

of murine ^{125}I-EGF binding to A431 cells was observed in the presence of the anti-MINT5 sera from protocols I and III (data not shown).

With both MAbs, the serum dilution at which 50% of Ab1 binding was inhibited was comparable with the titre observed in the Ab1 cross-linking assay (see Figure 1). The two sera of mice immunised with MRBC-MGR6-TM which were positive in Ab1 cross-linking assay were also able to compete ^{125}I-MGR6 binding to SKBr3 cells (data not shown).

Production of anti-Ab1 MAb

The immunised mice that exhibited the highest titre and competition ability from protocols I and III were used for generation of MAbs. With anti-MINT5 splenocytes, no positive supernatants were obtained from hybrids generated by either immunisation protocol. In addition, fusion of anti-MGR6 splenocytes from protocol I was unsuccessful. By contrast, four supernatants (2.4%) of 169 grown hybrids from protocol III showed reactivity with MGR6 in an Ab1 cross-linking assay. Of the four anti-Ab1 hybridomas (all IgG1), two (IdM6.1 and IdM6.4) after two subclonings were specific for Ab1 (MGR6), whereas IdM6.2 and IdM6.3 showed an equal or higher response against the isotype-matched unrelated MAb (Table 2). After purification from mouse ascites, only IdM6.4 inhibited MGR6 binding to SKBr3 cells (IC_{50} = 6.7 nM).

MAb MINT5 idiotype

To determine whether the recognition of an EGF-R binding site by MAb MINT5 prevents the development of an idiotypic cascade, we analysed the mimicry of murine EGF by the MINT5 idiotype. MINT5 recognition of murine EGF-R was assessed by competition assays on the EGF-R-positive murine cell line 3T3-Swiss and, as positive control, on human A431 tumour cells. On the human cell line, both murine and human ^{125}I-EGF (input 1.6 nM) were inhibited by human EGF with the same IC_{50} (2.8 ± 1.3 nM). As shown in Figure 4, cold human EGF and MAb MINT5 reduced murine ^{125}I-EGF binding to A431 cells to 22 and 15%, respectively, of the total bound in the absence of competitors. By contrast, human EGF but not MAb MINT5 blocked murine ^{125}I-EGF binding on 3T3-Swiss cells (37 and 92% residual murine ^{125}I-EGF binding, respectively).

To further analyse the specificity of MINT5 for the human EGF-R, the MAb pattern of reactivity was compared with that of murine EGF on normal murine epidermis and on the human solid tumour A431 by immunohistochemical staining of frozen sections from xenografts in athymic mice. MINT5 recognized only A431 cells (Figure 5c), whereas murine EGF reacted with both the epidermal tissue (Figure 5a) and the tumour mass (data not shown), irrespective of their origin.

Anti-MINT5 response in cancer patients

The immunogenicity of MINT5 was also analysed in a clinical setting. Six patients with lung carcinoma, entered in a phase I study to evaluate the toxicity and biodistribution of MINT5 MAb, were tested for development of HAMA and anti-idiotypic responses (Table 3). Only 1 patient developed a detectable HAMA response around 15 days after a single injection of the entire murine MAb. The response did not increase significantly at 30–40 days, and neither HAMA serum samples competed with the binding of MAb MINT5 to the relevant target cells A431. By contrast, murine MAb

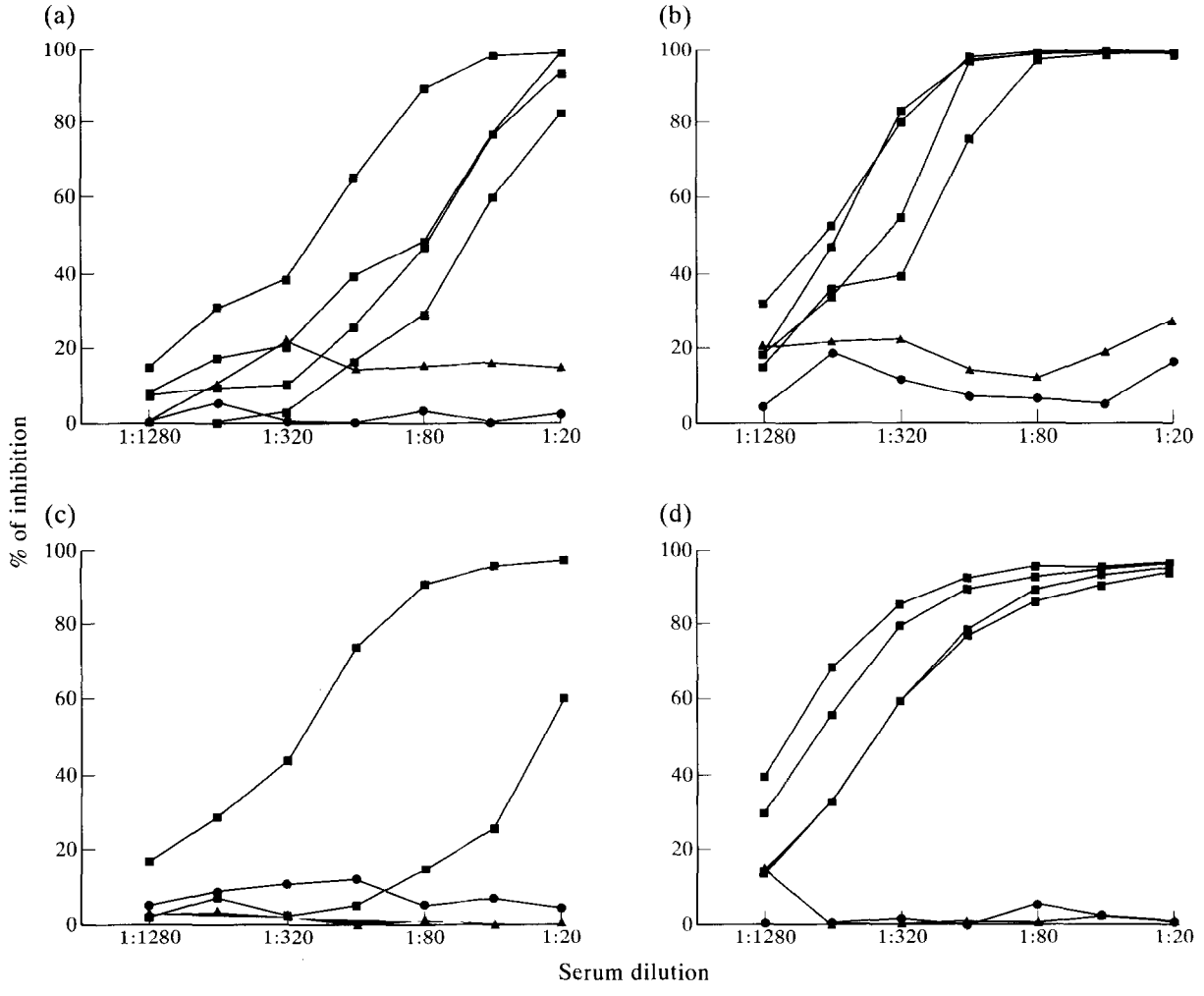

Figure 3. Specificity of the anti-Ab1 response. C57BL/6-positive sera from the last bleeding of protocols I and III were used to inhibit the binding of the corresponding ^{125}I-Ab1 to the relevant target cells: SKBr3 (a,b), A431 (c,d). (a) Sera of mice immunised with MRBC-MGR6 (protocol I); (b) sera of mice immunised with KLH-MGR6 (protocol III); (c) sera of mice immunised with MRBC-MINT5 (protocol I); (d) sera of mice immunised with KLH-MINT5 (protocol III). ■, immune sera; ▲, control serum; ●, anti-MINT5 (a,b) and anti-MGR6 serum (c,d) that showed the greatest inhibitory effect in the same protocol.

Table 2. Characteristics of anti-MGR6 MAb

Anti-Ab1 MAb	Ab1 cross-linking assay* (cpm)		Idiotype inhibition assay†	
	MGR6	MAD11	IC_{50}	% Inhibition‡
IdM6.1	2612 ± 531	408 ± 42	—	—
IdM6.2	1646 ± 40	1615 ± 545	—	—
IdM6.3	1787 ± 193	2456 ± 791	N.D.	N.D.
IdM6.4	30647 ± 2875	425 ± 31	6.7	92

*Undiluted hybridoma supernatants were incubated in microtitre plates coated with MGR6 or with MAD11 used as an allotype- and isotype-matched control. Binding was revealed by incubation with ^{125}I-MGR6. Values represent the mean ± S.D. of four or five different experiments. †^{125}I-MGR6 and purified anti-Ab1 MAb in titration were incubated with SKBr3 cells. IC_{50}, concentration (nM) able to compete 50% of ^{125}I-MGR6 binding. —, inhibition lower than 20% even at the maximal concentration tested. ‡At the maximal concentration tested (67 nM). N.D., not done.

MOV18, of the same allotype and isotype as MINT5, and previously shown to be highly immunogenic in a syngeneic Balb/c model [7], induced a high level of HAMA response in all 6 ovarian carcinoma patients examined. Thirty to 40 days after a single injection of a comparable dose of entire murine MAb, all patients had developed a vigorous HAMA response and in 3 patients, the positive sera were able to compete with the binding of MAb MOV18 to the relevant target cells IGROV1.

None of the patients entered in the two studies had detectable HAMA responses before treatment (data not shown).

DISCUSSION

Our aim in studying the immunogenicity of the idiotypes of two antigrowth factor receptor MAbs was to gain further information about the intrinsic properties of these MAbs in the context of their clinical use and possibly to generate Ab2 as useful tools in an experimental setting for analysis of the receptor–ligand interaction.

To analyse the immune stimulation induced only by idiotypic components, we initially used a syngeneic model successfully applied to other antitumour MAbs [5, 7, 8, 29,

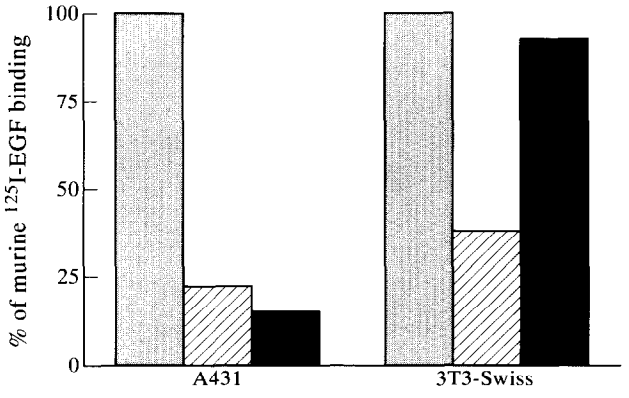

Figure 4. Recognition of murine and human EGF-R by MAb MINT5. A431 (3×10^4/well) and 3T3-Swiss (8×10^5/well) cells were incubated with 50 µl of murine ^{125}I-EGF (5×10^4 and 10^6 cpm, respectively) in the presence of a 100-fold molar excess of human EGF or MAb MINT5. The low level of murine EGF-R expression necessitated the use of a higher number of 3T3-Swiss cells for a detectable signal. Results are expressed as percentage of bound murine ^{125}I-EGF in the absence of inhibitors. ▩, no inhibitor; ▨, human EGF; ■, MAb MINT5.

Table 3. HAMA response after a single injection of murine MAb in cancer patients

MAb treatment	Total response (ng/ml)* 15–20 days	30–40 days	Anti-idiotype response†
MINT5			
1	—	—	N.D.
2	54	62	no
3	—	—	N.D.
4	—	—	N.D.
5	—	—	N.D.
6	—	—	N.D.
MOV18			
7	151	N.D.	no
8	N.D.	228	no
9	N.D.	>3200	yes
10	—	285	yes
11	>3200	>3200	yes
12	N.D.	>3200	no

*Evaluated by an ELISA-HAMA kit. —, below the level of detection (5 ng/ml). †Evaluated by idiotype inhibition assay. The response was considered positive when more than 50% inhibition was achieved with 1:20 serum dilution. N.D., not done.

30]. However, in the absence of an immunogenic carrier, unlike our previous observations with MAb MOV19 [31] or MOV18 [7], Balb/c mice did not mount a response against the idiotype of the syngeneic MAb MGR6 or MINT5. The use of allogeneic mice revealed a similar trend in the development of an anti-Ab1 response with both MAbs, although the number of mice for each immunisation protocol was too small to draw definitive conclusions. An autologous carrier elicited a detectable titre of circulating antibodies, but the total anti-Ab1 responses as well as their anti-idiotype components appeared sooner and at higher levels using the xenogeneic carrier KLH. A similar and vigorous anti-KLH response was observed in MGR6- and MINT5-immunised animals, whereas anti-isotypic and -allotypic responses were induced only by MAb MGR6. Despite the presence of an anti-idiotypic response in mice immunised with either MAb, only the fusion of splenocytes from the MGR6-KLH protocol led to the generation of anti-Ab1 MAb. The low frequency of hybridomas secreting anti-Ab1 MAb is consistent with previous data [32] and might be explained by hyperimmunisation of mice, which stimulates B cells located outside the spleen while inducing suppression of spleen B cells [33].

Ab2 are generally classified into four types (Ab2α, Ab2β, Ab2γ and Ab2ε) according to their specificities for different regions of the target antibody. Only Ab2β and Ab2γ bind to idiotopes associated with the antigen-binding site (paratope) of the original antibody (Ab1). In the case of Ab2β, its paratope mimics the original epitope sufficiently to display similar biological activity and immunogenicity. One of the four anti-MGR6 MAb produced, IdM6.4, inhibited the binding of MGR6 MAb on breast carcinoma cells. Further characterisation of IdM6.4 reactivity indicates that it recognises only bivalent but not monovalent fragments of MGR6 already bound to the relevant receptor (manuscript in preparation), suggesting that IdM6.4 recognises an idiotope in or near the antigen-combining site. Formal proof that IdM6.4 mimics the HER-2/neu binding site awaits the demonstration that it induces an Ab1-like response. Independently of its γ or β nature, the Ab2 IdM6.4 appears to be useful in the identification and purification of the Ab1 or its derivatives (e.g. chimeric MAb, single chain Fv and immunotoxins). If IdM6.4 is an Ab2β, it could also represent an excellent research tool in clarifying the role of different HER-2/neu ligands [34].

The cross-competition between MAb MINT5 and human and murine EGF on A431 cells (Figure 4, [21]) raised the possibility that the MAb recognises the receptor binding site. Analysis of the three-dimensional structure of murine [35] and human [36] EGF by proton nuclear magnetic resonance, and the use of synthetic peptides [37] has served to identify a conserved binding site (residues 20–31) in the two ligands. Consistent with these results, we observed that human EGF inhibits binding of murine EGF to human and murine receptors. Thus, the anti-Id component of an anti-MINT5 response might be directed against both murine and human EGF. Since the anti-MINT5 sera had a high specificity for the MAb idiotype but did not inhibit murine EGF binding to human EGF-R on A431 cells, and since MINT5 did not recognise the murine EGF-R, our data suggest that MINT5 interacts with an epitope that partially overlaps with the ligand-binding domain of the human EGF-R, but not with the corresponding domain on the murine receptor. Alternatively, the high specificity of the murine immune response which appears to be directed only against the variable regions of MINT5, provides the rationale for further efforts to generate Ab2 MAb for biotechnological applications.

In a preliminary clinical study in lung carcinoma patients, MINT5 was apparently unable to induce a HAMA response. By contrast, sera of patients entered in another clinical trial and injected with comparable doses of another murine IgG1 MAb, MOV18, were analysed simultaneously with the same kit and were found to exhibit a high level HAMA response. Although we cannot exclude that the observed difference in MAb immunogenicity reflects a difference in the immune status of the patients due to the disease and/or previous chemotherapy regimens, an extensive survey of the literature [38] indicates that most patients with solid tumours are immunocompetent and that, independently of the immunisation

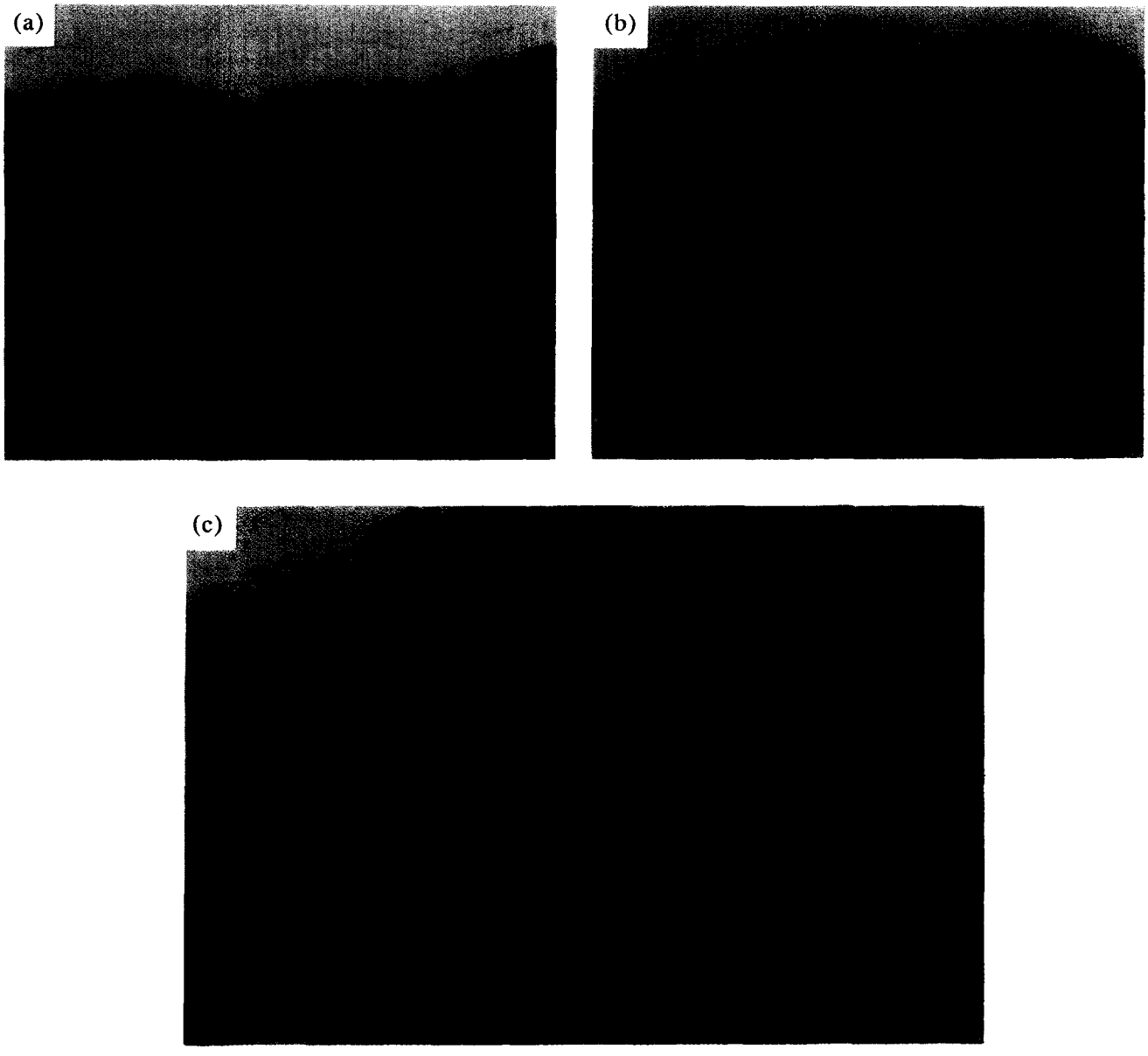

Figure 5. Photomicrographs of cryostat sections immunostained with MAb MINT5 and murine EGF and detected using ABC complex and diaminobenzidine (see Materials and Methods). (a) Biotinylated EGF on murine epidermal tissue (×400). (b) Negative control on murine epidermis (×400). (c) Biotinylated MAb MINT5 on s.c. solid tumour A431 grown in an athymic mouse (×1000).

schedule, a HAMA response is inducible by murine MAb. Moreover, some murine MAb became more immunogenic and the induced response was found to be directed mainly to their variable regions. In fact, the use of chimeric or humanised MAb does not appear to completely abrogate the production of anti-idiotypic Abs [38].

In conclusion, our animal data together with our preliminary results in humans suggest some similarity in the anti-Ab response of the two species. We speculate that immunogenicity of an MAb rests, at least in part, in its sequence and is therefore an intrinsic property of the MAb. Further studies in different animal species, as well as phase I and II clinical trials, will shed light on this hypothesis.

1. Jerne NK. Towards a network theory of the immune system. *Ann Immunol* 1974, **125**, 373–389.
2. Cazenave P. Idiotypic-anti-idiotypic regulation of antibody synthesis in rabbits. *Proc Natl Acad Sci USA* 1977, **74**, 5122–5125.
3. Nisonoff A. Idiotypes: concepts and applications. *J Immunol* 1991, **147**, 2429–2438.
4. Greenspan NS, Bona CA. Idiotypes: structure and immunogenicity. *FASEB J* 1993, **7**, 437–444.
5. Kusama M, Kageshita T, Chen ZJ, Ferrone S. Characterization of syngeneic antiidiotypic monoclonal antibodies to murine anti-human melanoma high molecular weight melanoma-associated antigen monoclonal antibodies. *J Immunol* 1989, **143**, 3844–3852.
6. Bhattacharya-Chatterjee M, Mukerjee S, Biddle W, Foon KA, Kohler H. Murine monoclonal anti-idiotype antibody as a poten-

tial network antigen for human carcinoembryonic antigen. *J Immunol* 1990, **145**, 2758–2765.
7. Miotti S, Mantovani L, Pupa SM, Bellosta P, Colnaghi MI. Characterization of two anti-idiotypic monoclonal antibodies raised against the anti-tumor monoclonal antibody MOv18. *J Immunol Res* 1989, **1**, 59–65.
8. Pupa SM, Bazzini P, Ménard S, Colnaghi MI. Network of idiotypic and anti-idiotypic antibodies related to the ovarian carcinoma-associated folate binding protein. *Anticancer Res* 1992, **12**, 1565–1570.
9. Chen ZJ, Yang H, Liu CC, Hirai S, Ferrone S. Modulation by adjuvants and carriers of the immunogenicity in xenogeneic hosts of mouse anti-idiotypic monoclonal antibody MK2-23, an internal image of human high molecular weight-melanoma associated antigen. *Cancer Res* 1993, **53**, 112–119.
10. Mittelman A, Chen ZJ, Kageshita T, *et al.* Active specific immunotherapy in patients with melanoma. *J Clin Invest* 1990, **86**, 2136–2144.
11. Mittelman A, Chen ZJ, Yang H, Wong GY, Ferrone S. Human high molecular weight-melanoma associated antigen (HMW-MAA) mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: induction of humoral anti-HMW-MAA immunity and prolongation of survival in patients with stage IV melanoma. *Proc Natl Acad Sci USA* 1992, **89**, 466–470.
12. Herlyn D, Wettendorff M, Schmoll E, *et al.* Anti-idiotype immunization of cancer patients: modulation of the immune response. *Proc Natl Acad Sci USA* 1987, **84**, 8055–8059.
13. Steplewski Z, Fox K, Glick J, Koprowski H. Human antimouse antibody response in patients treated with multiple infusions of monoclonal antibody CO17-1A. In Vaeth JM, Meyer JL, eds. *The Presence and Future Role of Monoclonal Antibodies in the Management of Cancer. Ther Radiat Ther Oncol*, Vol. 24. Basel, Karger, 1990, 69–72.
14. Mittelman A, Chen ZJ, Liu CC, Hirai S, Ferrone S. Kinetics of the immune response and regression of metastatic lesions following development of humoral anti-high molecular weight-melanoma associated antigen immunity in three patients with advanced malignant melanoma immunized with mouse antiidiotypic monoclonal antibody MK2-23. *Cancer Res* 1994, **54**, 415–421.
15. Ferrone S, Chen ZJ, Liu CC, Hirai S, Kageshita T, Mittelman A. Human high molecular weight-melanoma associated antigen mimicry by mouse anti-idiotypic monoclonal antibodies MK2-23. Experimental studies and clinical trials in patients with malignant melanoma. *Pharmac Ther* 1993, **57**, 259–290.
16. Aaronson SA. Growth factors and cancer. *Science* 1991, **254**, 1146–1153.
17. Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *Br Med Bull* 1991, **47**, 87–98.
18. Volm M, Efferth T, Mattern J. Oncoprotein (c-myc, c-erbB1, c-erbB2, c-fos) and suppressor gene product (p53) expression in squamous cell carcinomas of the lung. Clinical and biological correlations. *Anticancer Res* 1992, **12**, 11–20.
19. Slamon DJ, Godolphin W, Jones LA, *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989, **244**, 707–712.
20. Rilke F, Colnaghi MI, Cascinelli N, *et al.* Prognostic significance of HER-2/*neu* expression in breast cancer and its relationship to other prognostic factors. *Int J Cancer* 1991, **49**, 44–49.
21. Tosi E, Valota O, Negri DRM, *et al.* Antitumor efficacy of an anti-epidermal growth factor receptor monoclonal antibody and its F(ab')2 fragment against high and low EGFR-expressing carcinomas in nude mice. *Int J Cancer* 1995, **62**, 643–650.
22. Tagliabue E, Centis F, Campiglio M, *et al.* Selection of monoclonal antibodies which induce internalization and phosphorylation of $p185^{HER2}$ and growth inhibition of cells with HER2/*neu* gene amplification. *Int J Cancer* 1991, **47**, 933–937.
23. Centis F, Tagliabue E, Uppugunduri S, *et al.* p185 HER2/neu epitope mapping with murine monoclonal antibodies. *Hybridoma* 1992, **11**, 267–276.
24. Miotti S, Canevari S, Ménard S, *et al.* Characterization of human ovarian carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. *Int J Cancer* 1987, **39**, 297–303.
25. Marchalonis JJ. An enzymatic method for the trace iodination of immunoglobulin and other proteins. *Biochem J* 1969, **113**, 299–305.
26. Gullick WJ, Downward J, Waterfield MD. Antibodies to the autophosphorylation sites of the epidermal growth factor receptor protein-tyrosine kinase as probes of structure and function. *EMBO J* 1985, **4**, 2869–2877.
27. Ménard S, Tagliabue E, Canevari S, Fossati G, Colnaghi MI. Generation of monoclonal antibodies reacting with normal and cancer cells of human breast. *Cancer Res* 1983, **43**, 1295–1300.
28. Crippa F, Bolis G, Seregni E, *et al.* Single dose intraperitoneal radioimmunotherapy with the murine monoclonal antibody 131I-MOv18: clinical results in patients with minimal residual disease of ovarian cancer. *Eur J Cancer* 1995, **31A**, 686–690.
29. Viale G, Grassi F, Pelagi M, *et al.* Anti-human tumor antibodies induced in mice and rabbits by "internal image" anti-idiotypic monoclonal immunoglobulins. *J Immunol* 1987, **139**, 4250–4255.
30. Chen ZJ, Yang H, Ferrone S. Human high molecular weight melanoma-associated antigen mimicry by mouse antiidiotypic monoclonal antibody MK2-23: characterization of the immunogenicity in syngeneic hosts. *J Immunol* 1991, **147**, 1082–1090.
31. Colzani E, Pupa SM, Bazzini P, *et al.* Study of the suitability of the immunological approach to detect new oncogene products. *J Immunol Res* 1992, **4**, 73–78.
32. Li J-L, Chen J-L, Ouyang M-H, *et al.* Immunization strategies for the production of rat monoclonal anti-idiotope antibodies. *J Immunol Methods* 1991, **142**, 15–20.
33. Micheel B, Scharte G. Multiple antigen injections decrease the yield of hybridomas producing monoclonal antibodies. *Hybridoma* 1993, **12**, 227–229.
34. Dougall WC, Qian X, Peterson NC, Miller MJ, Samanta A, Greene MI. The *neu*-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene* 1994, **9**, 2109–2123.
35. Montelione GT, Wüthrich K, Nice EC, Burgess AW, Scheraga HA. Identification of two anti-parallel b-sheet conformations in the solution structure of murine epidermal growth factor by proton magnetic resonance. *Proc Natl Acad Sci USA* 1986, **83**, 8594–8598.
36. Cooke RM, Wilkinson AJ, Baron M, *et al.* The solution structure of human epidermal growth factor. *Nature* 1987, **327**, 339–341.
37. Komoriya A, Hortsch M, Meyers C, Smith M, Kanety H, Schlessinger J. Biologically active synthetic fragments of epidermal growth factor: localization of a major receptor-binding region. *Proc Natl Acad Sci USA* 1984, **81**, 1351–1355.
38. Khazaeli MB, Conry RM, LoBuglio AF. Human immune response to monoclonal antibodies. *J Immunother* 1994, **15**, 42–52.

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