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SINCE KÖHLER AND MILSTEIN described the technique to isolate monoclonal antibodies (MoAbs), much effort has been focused to isolate MoAbs reactive with tumor antigens for diagnostic and therapeutic applications. Despite intense research, the isolation of clinically relevant MoAbs has been disappointing. Preferably, such MoAbs are reactive with tumor-specific antigens (TSA) which should be expressed by all tumor cells of a certain tumor type. However, equivocal evidence for unique TSA in human malignancies is still lacking. Most human malignancies have weak, if any, detectable immunogenic properties, indicating that most human cancers either are nonimmunogenic or mediate strong immunomodulatory effects. Current MoAbs of interest recognize tumor-associated antigens (TAA) which are either differentiation antigens (transiently) expressed during organogenesis, or aberrantly expressed antigens, which are (transiently) expressed elsewhere in nonrelated normal tissue(s).

Administration of MoAbs that react with an antigen present on many normal tissues may result in unwanted side effects and diminish the amount of MoAb delivered to the tumor, influencing the efficacy of MoAb treatment. Therefore, 1) little cross-reactivity with nontumorous tissues is preferable; 2) the TAA density on the tumor cells should be high to achieve sufficient accumulation of MoAb; and 3) all tumor cells must express the TAA to enable the MoAb to specifically target to all tumor sites, eg, to accomplish visualization of all tumor sites or to achieve complete tumor remission. Unfortunately, for the vast majority of TAA that have been defined by anticarcinoma MoAbs, these criteria are not met: 1) the TAA is usually expressed on more than one type of carcinoma; 2) not all tumors within a given tumor type express the TAA (inter-tumor heterogeneity); 3) not all cells within a tumor lesion, including metastases, express a particular TAA (intratumor heterogeneity); 4) lesions of one patient express a TAA to a varying degree; 5) temporal modulation of TAA may occur; and 6) TAA expressed on most cells of a particular carcinoma are often expressed on some normal adult tissues. In general, with higher percentage of positive tumor cells and tumors expressing the TAA, cross-reactivity with normal tissues increases.

MOABS REACTIVE WITH RENAL-CELL CARCINOMA

A number of MoAbs reactive with cell surface antigens of renal-cell carcinoma (RCC) have been identified. The specificity of the majority of RCC-related MoAbs has been established using immunohistochemistry. Less is known about their targeting abilities and antitumor efficacy. The MoAbs can be divided into two groups: 1) MoAbs recognizing differentiation antigens, and 2) MoAbs recognizing RCC-TAA aberrantly expressed in RCC, ie, recognizing an RCC-antigen absent from normal kidney. Most MoAbs of the latter category show very restricted cross-reactivity with normal tissues as judged using immunohistochemistry. It appears that these MoAbs identify different RCC-TAA because they display different cross-reactivity with normal tissues. In view of their restricted cross-reactivity with normal tissues in combination with expression of a given RCC-TAA in most RCC, these MoAbs are prime candidates for clinical investigations.

One of the MoAbs recognizing an aberrantly expressed RCC-TAA, MoAb G250, was obtained after fusion of spleen cells from a mouse immunized with fresh RCC homogenates. This MoAb identifies an antigen absent from normal kidney and other normal tissues examined, with the exception of gastric mucosal cells and cells of the larger bile ducts. In the initial study, G250 antigen expression of 55 RCC was examined: 42 of 47 primary RCC showed homogeneous G250 antigen expression (89%), four tumors showed heterogeneous G250 antigen expression and only one primary tumor was completely devoid of G250 antigen expression. More importantly, of eight metastases examined,

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five showed homogeneous G250 expression (62%), two showed heterogeneous G250 expression and one metastasis failed to express G250 antigen. With an increased number of RCC tested, no difference with respect to the percentage of RCC expressing G250 antigen has been observed. In 77 of 95 (81%) primary RCC cases that were investigated, more than 50% of tumor cells were scored positive and in an additional eight patients, primary tumor cells also stained, albeit with lower frequency; ie, approximately 90% of primary RCC express G250 antigen. For the metastatic lesions, G250-positive tumor cells were noticed in 18 of 22 tumors (82%) examined, with homogeneous staining in 9 of 17 metastatic RCC examined (53%). In general, clear cell RCC tend to show homogenous G250 antigen expression, whereas non-clear cell RCC show heterogenous G250 expression. In view of the restricted cross-reactivity with normal tissues and the homogeneous expression in most RCC examined, this MoAb seemed useful as a therapeutic and/or radiodiagnostic agent.

**EXPERIMENTAL DATA**

**Tumor Targeting Studies**

The preferential accumulation of MoAb G250 was investigated in a RCC xenograft model. BALB/c nu/nu mice were xenografted with human RCC and/or human non-RCC tumors, and injected with 125I-labeled MoAb G250 IgGl, F(ab')2 or Fab'. Specific MoAb G250 accumulation was observed in the RCC tumors for all antibody forms. G250-negative tumors did not show any MoAb G250 uptake, nor did the G250-positive tumors show increased uptake of nonrelevant immunoglobulin. Higher uptake was generally found with intact G250 than with G250 F(ab')2 or Fab' fragments, most probably because of slower blood clearance, leaving the antibody more time to diffuse to the tumor cells. The lowest uptake was observed for Fab' fragments, related to a short biological half-life and lower avidity. Similar studies were performed by Chiou et al with MoAb A6H, an MoAb that reacts with a normal kidney-differentiation antigen. For MoAb G250 as well as MoAb A6H relatively high tumor/blood ratios were observed compared with other mouse targeting studies. This is possibly related to a general increase in permeability of the vascular bed in RCC xenografts.

In addition to these mouse-targeting studies, supplementary studies were performed with ex vivo perfused tumor-bearing human kidneys. In this model system tumor-bearing kidneys are flushed with preservation fluid immediately after surgery, whereupon the kidneys can be perfused with the MoAb of interest. Using this model system, the tumor targeting ability of 99mTc-labeled MoAb G250 was investigated. 99mTc MoAb G250 imaging of tumor-bearing kidneys resulted in clear images of RCC, with no MoAb G250 uptake in normal renal tissue. Despite the low temperature (0 to 4°C to assure appropriate pressures) and the relatively short circulation time (16 hours), tumor to kidney ratios were approximately 8:1. The preferential uptake of MoAb G250 in these ex vivo experiments were a clear indication that this MoAb held promise for clinical use.

**MoAb G250 Mediated Therapy**

Tumor cells may be lysed by MoAb-directed effector cells, so-called antibody-dependent cell-mediated cytolysis (ADCC). Several cell types have been shown to be active in ADCC, including macrophages, natural killer (NK) cells, killer (K) cells, and neutrophils. Experimental studies have highlighted the importance of antibody subclass, and mouse MoAbs of the IgG2a subclass have been shown to have the greatest activity in ADCC assays.

The antitumor effect of MoAb G250 alone and in combination with biological response modifiers (BRMs) was tested in a nude mouse model. Because the original MoAb G250 clone produced immunoglobulin of IgGl subclass, a poor mediator of ADCC, an IgG2a class switch variant of the original clone was developed. As a consequence, MoAb G250 specificity, idiotype and avidity were retained. Mice carrying established subcutaneous RCC tumors of approximately 50 mm3 were treated with different doses. Administration of 100 μg MoAb G250 per mouse for 6 weeks with three weekly injections resulted in a significant (P < .01) inhibition of mean tumor growth to 50% of control tumors. Increasing the MoAb G250 dose to 500 μg/dose, showed an inhibition of mean tumor growth to 35% of controls (P < .0001 versus control group). However, this increased tumor growth inhibition was not statistically different from the 100-μg dose schedule. No complete tumor regression was observed in any of the treated animals.
For this RCC xenograft (NU12), the optimal treatment schedule with biological response modifiers had been established in earlier studies. BRM treatment alone resulted in significant tumor growth inhibition, but complete tumor regression was never observed, despite the intense treatment schedule. We combined the best BRM treatment, rIFNa with rTNFa, with MoAb G250 treatment to investigate whether the efficacy of this combined treatment was superior to either treatment alone. This combination treatment significantly reduced NU12 tumor growth as compared with MoAb G250 or rIFNa/rTNFa alone (P < .0006 versus MoAb G250 alone, P < .003 versus rIFNa plus rTNFa). In the majority of mice tumors stabilized, and in several mice complete tumor regression was observed. No effects were observed when mice were treated with irrelevant IgG or with MoAb G250 F(ab')2 fragments, indicating that the effect was MoAb G250 specific and Fc-dependent. The Fc-dependency also indicated that ADCC was probably playing a role in the destruction of tumor cells.

Histochemical examination revealed that MoAb G250- or rIFNa/rTNFa-treated tumors were infiltrated by very few macrophages, if any, similar to control tumors in which macrophage infiltrates were absent. In contrast, massive macrophage tumor infiltration was observed in MoAb G250/ rIFNa/rTNFa-treated tumors. These infiltrates seemed to be infiltrating viable tumor nests and surrounded large necrotic areas. Most likely, BRM treatment leads to activation of mouse NK cells and macrophages, both mediators of ADCC, resulting in effective macrophage-mediated ADCC.

Although these results are encouraging, it remains to be investigated whether unmodified MoAb G250 IgG2a will be useful therapeutically. We have performed in vitro ADCC experiments using fresh isolated human monocytes as effector cells, and have not observed any tumor cell lysis. Different RCC cell lines were used to circumvent problems that may occur because of the use of a lysis-resistant cell line, and to assure that antigen density (which has been shown to be important in active ADCC) did not play a role. MoAb G250 IgG2a may not be effectively recognized by these human effector cells or, alternatively, these effector cells may have to be activated to mediate ADCC or G250-antigen density is too low. The latter possibility seems unlikely in view of the high number of antibody binding sites on these RCC cell lines. Enhancement of the ADCC capability of MoAb G250 can probably be achieved by chimerization of the antibody (chimeric MoAbs consist of mouse Ig variable regions grafted onto human Ig constant regions). However, preliminary ADCC experiments with chimeric G250 IgG1 and purified human monocytes as effector cells have been unsuccessful. Combining activated human lymphocytes with chimeric MoAb G250 may facilitate ADCC.

**CLINICAL EXPERIENCE**

Significant clinical experience with radiolabeled murine MoAbs that detect TAA has been acquired in the last decade. Radioimmunoscintigraphy studies of RCC with MoAbs have been limited, generally restricted to animal models. Vessella et al performed an imaging/radiotherapy trial in RCC patients with MoAb A6H, an MoAb that recognizes a kidney differentiation antigen. Positive images were obtained in 5 of 15 patients examined. The low number of positive images increased with an altered dosing schedule, but the number of imaged lesions remained unsatisfactory.

Based on our targeting studies in RCC-bearing mice and in ex vivo perfused tumor-bearing kidneys, a phase I study with 131I-labeled MoAb G250 was performed. In this protein dose escalation study, the primary study objectives were evaluation of the toxicity, pharmacokinetics, and localization capabilities of 131I MoAb G250. More than 90% of primary and metastatic disease as demonstrated by magnetic resonance imaging (MRI) and computed tomography (CT) scans was imaged by 131I MoAb G250. Metastatic lesions in lymph nodes, bone, and lung were visualized. Furthermore, additional metastatic disease documented at surgery but not detected by MRI and CT scans was visualized. For example, radioimmunoscintigraphy with 131I MoAb G250 showed a small hot spot in the liver of one of the patients which was not visualized by other means. Nine months later, the patient showed recurrence of RCC in that precise location. 131I MoAb G250 scanning also revealed diffuse metastatic RCC not recognizable by MRI or CT in a polycystic kidney of another patient.

From the ability of MoAb G250 to yield sharp images and visualize small tumor lesions it was
concluded that MoAb G250 has considerable potential as an imaging agent. However, not all primary or metastatic RCC lesions express G250 antigen, and some express it only in a minority of cells. This emphasizes the need for additional RCC-specific MoAbs because problems of antigen heterogeneity have to be overcome for therapeutic efficacy.

The absorbed dose to the tumor delivered by the best MoAb/radionuclide combination studies thus far has been calculated at 2,000 to 3,000 cGy, while maintaining less than grade IV toxic effects. Vaughan et al calculated the minimal requirement for effective therapy with 131I and 90Y-labeled MoAbs injected intravenously assuming an average accumulation of 0.005% of the administered dose/g of tumor and a maximum reasonable whole body dose of 2 Gy. These investigators concluded that the tumor uptake should be increased by a factor 10 for effective therapy. Calculation of the maximal fraction of the injected dose 131I MoAb G250 recovered in tumor sites showed that this was generally 10 to 100 times greater than previously reported accumulation of radiolabeled MoAb in solid tumors. The mean accumulation of the administered dose was also approximately tenfold higher than previously reported, fulfilling the requirements of Vaughan et al.

Based on these findings, a phase I/II trial with escalating doses of 131I labeled to 10 mg of MoAb G250 was undertaken in groups of three patients with inoperable metastatic RCC. Thus far, 21 patients have been treated. The maximum administered dose has been 90 mCi/m². Targeting of radioactivity to all known sites of disease was seen in all G250-antigen positive patients (19 of 21). Elevation of hepatic enzymes was observed in 18 patients, starting approximately 10 days after treatment and returning to baseline by 3 weeks. This elevation is probably due to MoAb G250 accumulation in the liver. G250 antigen is expressed in the larger bile ducts, and examination of liver biopsy specimens in the phase I protein dose escalation trial revealed MoAb G250 accumulation in bile duct epithelium. However, the amount of MoAb G250 necessary to saturate the hepatic compartment was minimal, and estimated in the range of 200 μg. Nevertheless, this amount seems to be sufficient to induce mild liver toxicity. At the 75-mCi/m² dose level, one of six patients had reversible grade IV thrombocytopenia, with a nadir at 4 weeks. No other major toxicity has been observed. There have been no major responses, but it is encouraging that stable disease was noted in 11 patients up to 9 months postradioimmunotherapy, as these patients with disseminated inoperable RCC tend to do very poorly.

**CHIMERIZED MoAb G250**

Generally, administration of murine MoAbs to patients elicits a human antimouse antibody (HAMA) response. This HAMA response is often directed against the Fc part of the immunoglobulin and independent of the amount of MoAb, or administration route. In all sera obtained from MoAb G250–injected RCC patients, HAMA of the IgM and IgG subclasses were detected. For repeated administration, eg, multiple radioimmunotherapy or multiple treatment with naked antibody to induce ADCC, HAMA responses need to be minimized because circulation HAMA reduces the tumor uptake of MoAb at subsequent administration because of MoAb-HAMA cross-linking. With recombinant technology, mouse MoAb variable regions can be grafted onto human Ig constant regions, and these constructs can be subsequently transfected into mammalian cells, which now produce chimeric immunoglobulin. Substitution of the mouse Fc part by human Fc has the additional advantage that all Fc-related effector functions are now matching the human effector cells. Principally, the use of chimeric IgG should augment ADCC mediated tumor cell lysis, and unmodified chimeric MoAb might be suitable to destroy (minimal) residual disease. Chimerized antibodies are expected to be less immunogenic in man. We have produced chimeric MoAb G250 (IgG1 subclass) and are currently testing their efficacy in ADCC and will start a phase I protein dose escalation trial to investigate the pharmacokinetics and targeting ability of chimerized MoAb G250.

**G250-RELATED ANTI-IDIOTYPE ANTIBODIES**

In the preceding sections, the use of MoAb G250 as a passive immunotherapeutic agent was discussed. Another strategy is to use MoAbs to induce active immunization. In 1974, Jerne proposed that the immune system is at a steady state by an equilibrium of lymphocytic clones bearing complementary receptors. This equilibrium can be disturbed either by a foreign or self-antigen, resulting in a response which aims at the restoration of the balance. In this response, anti-antibodies (Ab2)
play a major role. This hypothesis has become known as the network theory and has been substantiated by many investigators. The critical feature of this theory is that the binding pocket of the antibody that determines its specificity elicits anti-idiotypic antibodies. Several so-called idiotypic determinants can be present on one immunoglobulin molecule. In some cases anti-idiotypic antibodies will mimic the original antigen. These so-called internal image Ab2 offer promise as "vaccines" to various infectious agents, and they might be used to induce immune responses to tumor antigens. One of the advantages of this appealing approach is the long-lasting protection that vaccination with TAA-mimicking MoAbs might achieve. Principally, the requirements of these internal image MoAbs are the same as those for MoAbs recognizing TAA that might be useful for passive immunotherapy. Cross-reactivity with normal tissues should be minimal, to minimize undesired side effects related to organ toxicity elicited by vaccination with internal image MoAbs resembling normal differentiation antigens. Chattopadhyay et al have demonstrated that vaccination with Ab2 resembling a melanoma-associated TAA that is expressed on a restricted number of normal tissues can induce TAA-specific responses, indicating that TAA expression on a restricted number of tissues does not hamper the successful implementation of Ab2 vaccination.

Considering the restricted expression of G250, vaccination with internal image Ab2 resembling this RCC-TAA might represent a new therapy for RCC patients. Therefore, we have started to isolate Ab2 bearing the internal image of MoAb G250. Six MoAbs were isolated after fusion of spleen cells from mice immunized with MoAb G250 which showed a dose-dependent inhibition of binding of 125I-MoAb G250 to antigen. Because Ab1-Ab2 interactions in close vicinity of Ab1-antigen interactions will interfere with Ab1-antigen binding, competition for antigen binding was insufficient evidence to conclude that true internal image Ab2 were isolated. To investigate whether these Ab2 could substitute for the initial G250 antigen, mice and rabbits were immunized with the Ab2 to monitor for the occurrence of Ab3 resembling MoAb G250 (so-called Ab1'). Indeed, for all six Ab2 Ab1' antibodies were detected, showing the functional identity of the six MoAbs and G250 antigen.

Initially, these six MoAbs were divided into two immunologically distinct groups, recognizing two partly overlapping epitopes. However, additional fine specificity studies have shown that the six internal-image MoAbs can be divided into four mutually exclusive groups, evidence that the six Ab2s recognize four slightly different idiotopes in the Ab1 binding pocket. Because they seem to recognize unique idiotopes in the MoAb G250 binding pocket, it implies that not all six internal image MoAbs are true representations of G250 antigen. Consequently, one of the MoAbs may show superior antitumor characteristics.

To investigate the antitumor efficacy of immunization with Ab2, serum of Ab2-immunized mice was transferred to nu/nu BALB/c mice challenged with human RCC cells. In this Winn-type assay, transfer of any Ab3-serum resulted in significant tumor growth inhibition (4 weeks; P < .05 versus all control groups, 8 weeks; P < .01 versus all control groups) and lower tumor take as compared with control groups (Table 1). Four weeks after tumor challenge, no significant difference between Ab3 treatment groups was seen. Small tumors were detectable in all Ab3-treated animals. However, 8 weeks after tumor challenge (3 weeks after cessation of therapy), remarkable differences between the different treatment groups were observed (Fig 1). All mice treated with Ab3-91 showed complete tumor regression. Macroscopically as well as microscopically no tumor remnants could be detected. Ab3-31 treatment resulted in tumor regression in the majority of animals and tumor stabilization of the remaining tumors. Similarly, Ab3-82 treatment effected tumor regression in the majority of animals, with extremely slow growing tumors in the remaining animals. After Ab3-71 treatment, slow growing tumors were measurable in all animals. The doubling time of tumors remaining after Ab3 treatment was significantly lengthened. Four weeks after cessation of therapy the tumors started to grow with doubling times comparable to control tumors. It is likely that 4 weeks after discontinuation of therapy the circulating levels of Ab1' antibody and serum factors were depleted and too low for continuation of tumor control. Likewise, the early growth of the tumors treated with Ab3-91 can be explained by a dose effect. Because the Ab1' titers were moderate, several treatments may be necessary to achieve sufficient high levels of Ab1' for tumor control and regression. Based on these results we conclude that NUH-91 immunization
elicit the most powerful antitumor effects, and that this Ab2 might be the best candidate for clinical trials.

Because the animals were treated with whole serum, the antitumor effects can also be explained by circulating serum factors, eg, T cell factors and cytokines. We and others have shown that combination therapy with BRMs, which are serum circulating factors, can result in significant tumor growth inhibition. Treatment of established RCC xenografts with interferon-alfa, tumor necrosis factor and MoAb G250 resulted in tumor growth inhibition and tumor regression.

However, preliminary experiments have shown that Ab3 treatment is superior to BRM/MoAbG250 treatment. It is therefore likely that the majority of the antitumor effects are Ab1 related. We are currently investigating the therapeutic efficacy of Ab3 serum in mice with established RCC xenografts.

The tumor regression induced by Ab3 serum transfer could also be explained by the presence of T cell factors such as specific macrophage arming factor (SMAF) and mast cell T-Cell factor (MTCF). These factors can, among others, provide antigen-specificity to nonspecific cells.

Immunization of immune competent mice with any of the six Ab2 investigated results in profound transferable antigen-specific cell-mediated immunity, with concomitant serum transferrable early delayed-type hypersensitivity responses, attributable to T cell factors.

Because these factors persist approximately 2 to 16 days after immunization, and animals were treated with immune serum harvested 7 days after immunization, part of the antitumor effects may have been

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**Table 1. Summary of Representative Protection Experiment With Ab3 Sera**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Mice</th>
<th>Tumor Take (%)</th>
<th>Tumor Volume (mm$^3 \pm$ SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 w</td>
<td>8 w</td>
</tr>
<tr>
<td>Ab3-31</td>
<td>5</td>
<td>100</td>
<td>80 9.8 $\pm$ 2.5* 11.8 $\pm$ 5.6†</td>
</tr>
<tr>
<td>Ab3-71</td>
<td>5</td>
<td>100</td>
<td>100 10 $\pm$ 2.2* 26.7 $\pm$ 5.2†</td>
</tr>
<tr>
<td>Ab3-82</td>
<td>6</td>
<td>100</td>
<td>66.7 8.1 $\pm$ 2.7* 17.9 $\pm$ 8.7†</td>
</tr>
<tr>
<td>Ab3-91</td>
<td>6</td>
<td>100</td>
<td>0 7.7 $\pm$ 2.2* 0†</td>
</tr>
<tr>
<td>Nontreatment</td>
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<td>100</td>
<td>100 23.5 $\pm$ 4.4 126 $\pm$ 16.6</td>
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<tr>
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<td>100</td>
<td>100 29 $\pm$ 7.5 98.9 $\pm$ 12.3</td>
</tr>
<tr>
<td>NMS§</td>
<td>5</td>
<td>100</td>
<td>100 31.8 $\pm$ 14.4 114 $\pm$ 21.0</td>
</tr>
</tbody>
</table>

*P < .05.
†P < .01 compared with last three cases in this list.
‡Abbreviation: NMS, treated with normal mouse serum.

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Figure 1. Individual tumor growth curves of RCC challenged mice treated with Ab3 sera. BALB/c nu/nu mice received s.c. injections of 10⁶ SK-RC-52 cells (G250-positive human RCC cell line) in the right flank, and treatment was started. Four groups of mice were treated with i.p. injections of NUH-Ab3 sera, and three groups served as controls: no treatment was given (None), mice were treated with serum from MOPC21-KLH immunized mice, or mice received normal mouse serum (not shown). Tumor volumes are expressed as mean ± SE. Figure is representative for several independently performed experiments.
elicited by T cell factors. It is likely that similar factors, with similar antitumor efficacy will be induced by Ab2 immunization of RCC patients.

**FUTURE PROSPECTS**

Our future efforts will be directed at investigating the targeting ability of chimerized MoAb G250. We anticipate that chimerization of murine MoAb G250 will allow multiple treatments, because human antimouse responses are reduced. If that is true, multiple treatments with, eg, high-dose 131I-labeled chimeric MoAb G250 would become feasible. Once the targeting ability and pharmacokinetics of chimeric MoAb G250 are known, we intend to investigate the radioimmunotherapeutic potential of high-dose 131I-labeled chimeric MoAb G250. In addition, chimerization may provide the antibody with appropriate effector functions. This will enable engagement of human effector cells with chimeric MoAb-G250 coated tumor cells, effectuating tumor cell lysis.

We will also pursue the possibilities of antidiotypic therapy. Much needs to be resolved with respect to the identification of the mechanism behind the observed antitumor efficacy. Currently, we are in the process of identifying which effector cells are involved in tumor cell elimination, and are investigating which factors are of prime importance for tumor regression. Regardless of the effector mechanism, our observation that Ab2 vaccination results in tumor regression is of clinical relevance because it seems to indicate that tumor immunity can be acquired. Patients at risk for tumor recurrence, eg, patients with T3NoMo disease, may benefit from adjuvant treatment by Ab2 vaccination.

**REFERENCES**