Specific and Nonspecific Immunoassays to Detect HAMA After Administration of Indium-111-Labeled OV-TL 3 F(ab')$_2$ Monoclonal Antibody to Patients with Ovarian Cancer

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The development of human anti-mouse antibodies (HAMA) may cause problems in radioimmunotargeting studies, but may also improve survival of patients. To identify the presence of HAMA in blood samples from patients intravenously injected with 1 mg of ^{111}In-labeled OV-TL3-F(ab')$_2$, we developed three specific OV-TL 3-based HAMA assays and tested these along with two commercially available nonspecific HAMA assays (Sorin and Immunomedics). The specific assays were positive for HAMA with 10 postinjection serum samples from 7 patients. Eight of the 10 samples were also HAMA positive with one or both nonspecific HAMA assays. Conflicting results were observed with half the number of samples. The two nonspecific assays also reacted positively with another 11 serum samples from 5 patients including their preinjection samples. Despite some contradictory results, the nonspecific HAMA assays identify both pre-existent and Mab-induced HAMA, whereas the specific OV-TL3-based HAMA assays identify specific immune-responses occurring after the OV-TL 3 injection.

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The development of human anti-mouse antibodies (HAMA) after the administration of murine monoclonal antibodies (Mab) against tumor-associated antigens in radioimmunotargeting studies may cause problems, which are probably the result of formation of high molecular mass complexes with the injected Mab (*1*). The formation of immune complex may lead to altered blood clearance, increased hepatic or splenic uptake and reduced tumor uptake of the radiolabeled Mab (*1,2*). Heterophilic antibodies, however, may also be present in the serum of normal healthy individuals not previously treated with Mab ("pre-existent HAMA") (*3,4,5*). Recently, it was suggested that the activation of the anti-idiotypic network due to development of anti-idiotypic HAMA after immunoscintigraphy may improve survival of patients with advanced ovarian carcinoma (*6,7*).

Our group has developed (*8*) and characterized (*9,10*) an ovarian carcinoma-associated murine Mab, OV-TL 3, which reacts with more than 90% of ovarian carcinomas (*8,11*). We have recently reported the diagnostic accuracy of immunoscintigraphy with the ^{111}In-labeled murine Mab OV-TL 3-F(ab')$_2$, administered as a single dose (1 mg) to patients suspected of having ovarian carcinoma (*12*).

In order to be able to anticipate the results of future immunotargeting studies, we assessed the existence or development of HAMA in these patients.

Price et al. (*13*) already suggested that Mab-induced HAMA can be more reactive with polyclonal IgG than with the Mab that was administered to the patient. In contrast, Turpeinen et al. (*14*) have suggested that the best specificity and sensitivity of HAMA assays is achieved by using the same Mab on the solid phase of the HAMA assay as that administered to the patient. Thus, the aim of the present study was:

- to determine whether patients in the diagnostic immunotargeting study demonstrate concentrations of pre-existent or Mab-induced HAMA significant enough to be detected with commercially available HAMA assay kits
- to develop several double determinant "sandwich" assays based on the injected Mab OV-TL 3 and its F(ab')$_2$
- to test whether these specifically designed HAMA assays allow for the identification of HAMA that may develop after injection of the "relevant" Mab
- to compare and assess the gain of these results with those obtained using the commercial HAMA assays
- to test whether the designed assays enable researchers to distinguish anti-isotypic from anti-idiotypic HAMA

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since, by definition, the latter type of heterophilic antibody cannot be detected with the more general commercial HAMA assays.

MATERIALS AND METHODS

Serum Samples

Serum samples were obtained from 33 patients suspected of having ovarian cancer. Prior to i.v. administration of 1 mg of ^{111}In-labeled OV-TL 3 F(ab')$_2$, a blood sample was taken from each patient. None of the patients had previously received murine Mab. During the period of clinical follow-up, blood was also taken at both three and six weeks after immunoscintigraphy (except from Patients 1, 18, 26 and 30, at 3 wk postinjection and from Patients 1, 18, 19, 28 and 30–33, at 6 wk postinjection). From seven patients, between one and five additional blood samples were collected between Weeks 7 and 32 postinjection.

Labeling the Monoclonal Antibody

The Mab OV-TL 3 as well as the F(ab')$_2$ fragment of OV-TL 3 were purified by affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) (*15*). We labeled these preparations with Eu^{3+}-N'-(p-isothiocyanatobenzyl) diethylenetriamine-N^1,N^2, N^3,N^3-tetra acetate, as described by Hemmilä et al. (*16*), resulting in the incorporation of three atoms of Eu per mole OV-TL 3 F(ab')$_2$, and of four atoms of Eu per mole OV-TL 3. Here we take advantage of recent developments indicating the widespread applicability in immunoassays of europium chelates as labels. These time-resolved fluoroimmunoassays make use of europium chelates as labels and require the addition of an enhancement solution (based on 2-naphthoyltrifluoroacetone, a β-di-ketone) to elicit the formation of a fluorescent europium chelate complex. The high quantum yield, large Stokes shift and extremely long fluorescence lifetime of the complex nearly eliminate any losses in sensitivity caused by fluorescence background and scatter (*17*).

Coating of the Microtitration Strips

The Mab OV-TL 3 or its F(ab')$_2$ fragment is adsorbed to the walls of polystyrene microtiter wells (Eflab Oy, Helsinki, Finland) by incubating overnight at 4°C, 250 μl of purified OV-TL 3 antibody or its F(ab')$_2$ (4.0 mg/liter) in Tris buffer (100 mmole/liter, pH 7.8, 154 mmole of NaCl and 7.5 mmole of NaN_3/liter) per well. After incubation, the strips are washed five times with wash solution (36 mmole of NaCl, 0.25 g of a preservative, Germall-II (Sutton Laboratories Inc., Chatham, NJ) and 0.05% of Tween 20 surfactant per liter), saturated overnight with 300 μl of bovine serum albumin (Sigma Chemical Co., St. Louis, MO) solution: 5.0 g of albumin, 100 mmole of Tris buffer (pH 7.8); again washed five times with wash solution, and stored in a humidified atmosphere at 4°C.

Time-resolved Immunofluorometric Assays (IFMAS) for Detection of HAMA

Two hundred microliters of serum sample diluted twofold with assay buffer (the albumin solution above), or 200 μl of assay buffer for zero-dose control are pipetted into the coated microtiter wells and incubated overnight at room temperature. The reaction mixture is aspirated and the wells are washed five times with wash solution using a 12-channel washer (Wallac Oy, Turku, Finland). In the second step, we add to each well 200 μl of Eu-labeled OV-TL 3, or its F(ab')$_2$ (0.2 mg/liter). After incubating the wells for 3 hr at room temperature, the contents of the wells are aspirated and the plates are washed nine times with wash solution. Finally, to each well, 200 μl of enhancement solution is dispensed (containing 1.0 g of Triton X-100 surfactant, 6.8 mmole of potassium hydrogen phthalate, 100 mmole of acetic acid, 50 μmole of tri-n-octyl-phosphinoxide, and 15 μmole of 2-naphtoyltrifluoroacetone per liter). The strips are shaken for 5 min on a microshaker (Dynatech, Zug, Switzerland) and after another 10 min the fluorescence is measured in a Model 1230 Arcus time-resolved fluorometer (Wallac Oy, Turku, Finland), using a 1-sec counting time and a 400-μsec time delay (*18*).

Table 1 depicts the various types of (time-resolved) IFMAs for detection of human anti-OV-TL 3 antibodies as used in the present study: Assays 1, 2a and 2b are homologous "sandwich"-type assays because they apply either the intact OV-TL 3 molecule (Assay 1), or its F(ab')$_2$ fragment (Assays 2a and 2b), both as the capture and the signal (Eu-labeled) Mab. In the cases of Assays 1, 2a and 2b, anti-isotypic (and anti-allotypic) HAMA will bind to an epitope (antigenic determinant) located on any constant region of the capture and signal Mab (or fragment) of these assays (thus both serving as haptens to the HAMA), while anti-idiotypic HAMA recognize idiotopes located within the hypervariable regions of the Mabs used in these assays.

Assay 2b is equal to Assay 2a, except for supplementation of the former with 10% normal nonimmune mouse serum (Organon-Teknika-Cappel, Turnhout, Belgium) to block binding of human antimouse antibodies of the anti-isotype-type. A positive result with the homologous OV-TL 3 related Assay 1 may indicate the presence of (OV-TL 3 induced) anti-isotypic or anti-idiotypic

TABLE 1
Design of the In-House OV-TL 3 Related Assays and the Commercially Available General Assays for the Detection of HAMA in Serum Samples

Assay no.	Capture antibody	Signal antibody
	In-House OV-TL 3 Related HAMA Assays	
1	OV-TL 3, whole molecule	OV-TL 3, whole molecule, Eu^{3+}-labeled
2a	OV-TL 3, F(ab')$_2$	OV-TL 3, F(ab')$_2$ Eu^{3+}-labeled
2b	Assay 2b similar to 2a, but 10% normal nonimmune mouse serum added	
	Commercially Available HAMA Assays	
3*	Mouse Mab (IgG_1), whole molecule	Anti-human IgG, whole molecule, HRP-labeled††
4a†	Mouse IgG, whole molecule	Mouse IgG, whole molecule, HRP-labeled††
4b	Assay 4b similar to 4a, but capture antibody replaced by: OV-TL 3 F(ab')$_2$	

* Assay 3: ETI-HAMAK Kit, Sorin Biomedica, Saluggia, Italy.
† Assay 4a: ImmuSTRIP HAMA Immunomedics Inc., Warren, NJ.
†† Horseradish peroxidase (HRP) conjugated to signal IgG.

HAMA. If the homologous OV-TL 3 $F(ab')_2$ related Assay 2a is positive, this excludes anti-isotypic HAMA that recognize an epitope located on the F_c portion of OV-TL 3. A subsequent negative result with Assay 2b indicates that binding of HAMA of the anti-isotype cannot be blocked, suggesting that the HAMA detected is not anti-idiotypic in nature.

Commercial Assays for HAMA Detection

Serum samples were also tested with two commercial HAMA assays: the heterologous double-determinant immunoenzymometric assay (IEMA) ETI-HAMAK (Sorin Biomedica, Saluggia, Italy, Assay 3) that identifies HAMA against murine IgG_1 of the human IgG class (by using 100 μl of a 100-fold diluted test sample) and the homologous IEMA ImmuSTRIP HAMA (Immunomedics, Inc., Warren, NJ; Assay 4a), which is the most broad-spectrum HAMA assay, having been designed to identify all human antibody classes against all mouse IgG subclasses (in 100 μl of a 10-fold diluted test sample). These assays were performed according to the manufacturer's protocols. To investigate whether the HAMA detected by Assay 4a have any relationship with OV-TL 3, we also modified Assay 4a into 4b (Table 1). Assay 4b is similar to Assay 4a, but the capture antibody of the latter assay is replaced by OV-TL 3 $F(ab')_2$ as is also used in Assays 2a and 2b.

Since no HAMA standard or reference material is available for all these tests, the results of our assays are semi-quantitative and, for this reason, are expressed in terms of time-resolved fluorescence cps, or absorbance units of the proportion bound.

Data Assessment

To explore the data obtained from all assays, we have primarily assessed the results from entire groups of sera in terms of specificity (i.e., the preinjection sera) and sensitivity (the two postinjection groups). We considered assay results of postinjection sera to be positive if the responses of these samples were above the cutoff value of a given assay. Cutoff values were established by calculating the mean responses plus twice the standard errors of the 33 serum samples, collected preinjection from all the patients

TABLE 2
The Serum Samples Giving Positive HAMA Responses at 3 and 6 Weeks Postinjection with the OV-TL 3 Related HAMA IFMA 1

Cut-off‡		HAMA responses* with assay number†						
Patient no.	Week	1 14.2	2a 13.0	2b 16.9	3 289	4a 500	4b 160	Sample group
6	3 wk	64.9	65.8	—	—	2064	1286	A.1
6	6 wk	26.9	38.8	—	—	1023	301	2
15	6 wk	22.2	17.8	—	2282	633	161	3
33	3 wk	16.6	20.9	—	502	550	197	4
21	6 wk	35.6	24.8	—	643	—	—	5
25	3 wk	16.0	20.1	—	622	—	—	6
29	3 wk	45.0	36.4	—	—	—	170	7
29	6 wk	39.7	35.1	—	—	524	—	8
20	3 wk	20.7	20.9	22.6	—	—	—	B.1
15	3 wk	86.6	95.7	26.4	2326	912	254	2
4	pre	15.5	—	—	330	—	—	C.1
	3 wk	—	—	—	311	—	—	2
18	pre	—	—	—	308	—	—	3
3	pre	—	—	—	—	1151	—	D.1
	3 wk	—	—	—	—	637	—	2
	6 wk	—	—	—	402	—	—	C.4
22	pre	—	—	—	—	1639	—	D.3
	3 wk	—	—	—	—	906	—	4
	6 wk	—	—	—	—	2056	—	5
28	pre	16.3	—	—	—	562	—	6
	3 wk	—	—	—	—	561	—	7

* HAMA responses of serum samples of the two postinjection groups (3 wk, n = 29; 6 wk, n = 25); those above the cutoff value are numerical; those less than or equal to cutoff value are indicated by "—".

† Assay numbers refer to Table 1.

‡ Cutoff values established as mean + 2 × standard error of the responses of 33 serum samples comprising the preinjection group. Cutoff values and positive responses in terms of cps × 10^3 in case of Assays 1, 2a and 2b, or absorbance units × 10^{-3} in case of Assays 3, 4a and 4b.

studied. For comparison, we have also assessed these results with the intraindividual changes of HAMA responses as determined with Assays 1 and 2a, in the blood samples collected consecutively from each patient following OV-TL 3 injection (i.e., in terms of post-/preinjection ratios of individual patients). If these changes in assay responses were 200% or higher, we arbitrarily considered them significantly increased.

RESULTS

The cutoff values of all the HAMA assays used are given in Table 2. The resulting specificities (i.e., the proportion of true-negative HAMA responses over total preinjection samples) of all the HAMA assays used ranged between 90%–100%.

From all the serum samples collected 3-wk (n = 29) and 6-wk (n = 25) postinjection, Table 2 summarizes the 10 serum samples (Groups A and B) from 7 patients who reacted positively (i.e., above cutoff), with Assay 1. These samples also demonstrated concordant positivity in the homologous OV-TL 3 $F(ab')_2$ related Assay 2a. Concordant with these results are the intraindividual changes of >200% of all the samples of Groups A and B with both Assays 1 and 2a. The eight sera of Group A give subsequent negative results with Assay 2b whereas the two sera comprising Group B remain positive with Assay 2b. This strongly favors the presence of anti-idiotypic HAMA in one case (sample B.1).

From all the samples contained in Groups A and B, the two commercial assays, Assays 3 and 4a, display positive and concordant results in only three cases (A.3, A.4, B.2), discordant results (positive or negative) in five cases (A.1, A.2, A.5, A.6, A.8) and are both negative in two cases (A.7, B.1), including the only OV-TL 3 induced anti-idiotypic HAMA response observed (B.1).

In addition to the postinjection sera comprising Groups A and B, Table 2 also shows that the general heterologous HAMA Assay 3 gives positive results with two preinjection sera (Patients 4 and 18) and with two postinjection sera (Patient 4, 3-wk postinjection, and Patient 3, 6-wk postinjection) of Group C (C.1-4). These sera were negative with the OV-TL 3 related Assays 1, 2a and 2b. Also, the other general homologous HAMA assay, Assay 4a, reacts positive with seven sera from Patients 3, 22 and 28 (Group D, Table 2). In all cases, these series include the sera taken preinjection.

In order to see whether the three timepoints taken for blood sampling (preinjection, 3 wk and 6 wk postinjection) were sufficient to evaluate the presence of pre-existent HAMA activity or the induction of HAMA activity, we retrospectively monitored the follow-up of the seven patients from which additional serum samples taken postinjection were available after Week 6 until Week 32. These samples were subjected to further monitoring with Assays 1, 2a and 2b. Figure 1 illustrates the follow-up profiles in the cases of Patients 6, 21 and 29. Patient 6 demonstrated a fast and strong anti-isotypic response reaching maximum values at 3 wk and fully declining to cutoff values at 10-wk postinjection. Patient 21 showed an increasing anti-isotypic response from 6 wk onward, reaching a maximum at 15 wk postinjection. Some anti-idiotypic reactivity was also demonstrated from 12 wk postinjection. Patient 29 demonstrated a clear anti-isotypic response from 3 wk postinjection onward. After Week 6, some anti-idiotypic response could be demonstrated. Furthermore, the results from the specific HAMA assays, Assays 1 and 2a, deviated after Week 10 postinjection.

DISCUSSION

In our intravenous radioimmunotargeting studies with mouse Mab OV-TL 3, the patient's response to the injected

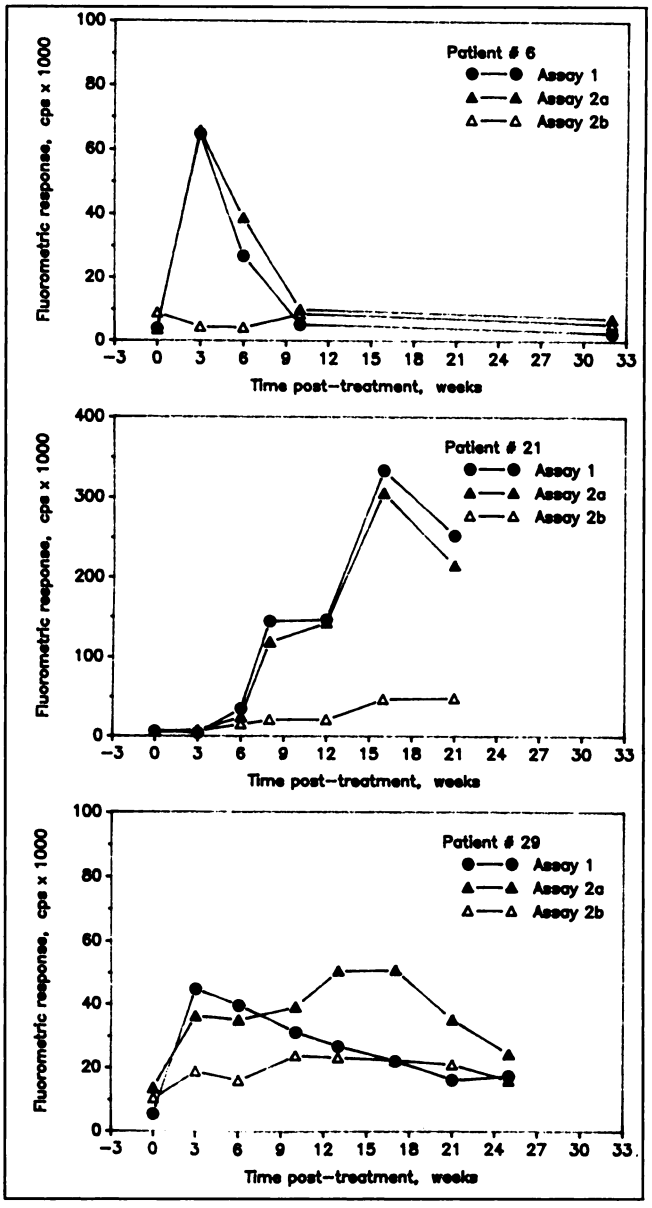

FIGURE 1. The follow-up profiles of Patients 6, 21 and 29 as monitored with Assays 1, 2a and 2b.

$F(ab')_2$ fragments is expected to be directed against constant or (hyper)variable domains of the IgG_1-OV-TL 3 molecule, and not against any part of the F_c portion of the molecule. Also, pre-existent HAMA may be present, and these antibodies might be reactive with epitopes on any domain in any (sub)class of mouse immunoglobulins. To investigate the occurrence of these immune responses, we have tested three different assays for the detection of HAMA, which develop after injection with mouse OV-TL 3 $F(ab')_2$. The results of three specific OV-TL 3-based assays have been compared with those of two more general HAMA assay systems, commercially available.

Comparison of the results obtained from entire groups of sera in terms of specificity and sensitivity with those of the intra-individual changes of HAMA responses in terms of postinjection-to-preinjection ratios of individual patients has shown that both approaches lead to the same conclusion regarding the detection of the specific OV-TL 3 related HAMA, developing after injection with Mab OV-TL 3 $F(ab')_2$.

Homologous OV-TL 3 Related HAMA Assays: Assays 1, 2a and 2b

The homologous OV-TL 3 specific HAMA Assays 1, 2a and 2b (Table 1), using 100 μl of sample, showed a postinjection anti-OV-TL 3 response in 10 samples from seven patients (Table 2, Groups A and B). The subsequent negative results with Assay 2b of the eight sera of Group A indicate that these samples contain OV-TL 3 induced HAMA of the anti-isotype, which recognizes epitopes located on the $F(ab')_2$ fragment. Since addition of normal mouse serum could block binding of HAMA in all samples except one (B.1) and only in part in another sample (B.2), these two samples are considered to contain a purely anti-idiotypic and a mixed form of anti-isotypic and anti-idiotypic HAMA response, respectively.

More General HAMA Assays: Assays 3, 4a and 4b

The two more general, non-OV-TL 3 related mouse IgG-based assays, Assays 3 and 4a, operate with sample volumes (100-fold and 10-fold diluted 100 μl samples, respectively) well below the 100 μl required by the homologous anti-OV-TL 3 assays, Assays 1, 2a and 2b. This may suggest that the more general assays yield at least equal or better analytical sensitivities than those obtained with all anti-OV-TL 3 assays tested. The heterologous Assay 3 (Sorin) identifies HAMA against mouse IgG_1 of the human IgG class, while the homologous Assay 4a (Immunomedics) recognizes all human antibody classes against all mouse IgG subclasses; the latter thus being the most broad-spectrum HAMA assay. However, apart from the obvious differences in specificity between the two non-specific assays to detect HAMA of different origin (pre-existent, Mab-induced, Mab-related, etc.), specificity is further complicated by the occurrence of allotypical and isotypical differences present between the mono- and polyclonal immunoglobulins used by these assays (*19*).

This is in line with the data of Price et al. (*11*) who demonstrated that removal of (anti-CEA induced) HAMA interference in a "sandwich"-type CEA assay was more effective with a polyclonal mouse IgG (whole mouse serum) or a mixture of monoclonal IgG subclasses than with an individual monoclonal IgG subclass added to the assay system. (Their explanation is that any HAMA response is polyclonal, and that some of the heteroclitic antibodies thus produced have a higher affinity for other IgG isotypes.)

Indeed, the combined use of the non-OV-TL 3 related Assays 3 and 4a, in fact only detects anti-IgG-isotype human antibodies, being seven of the sera of Group A (A.1-6, A.8) and one (B.1) out of two in Group B (Table 2). However, to address whether there is a relationship between the results of the two types of assays, a comparison with the results of the OV-TL 3 specific assays demonstrates that the two nonspecific assays are positive and concordant in just three cases (A.3, A.4, B.2) and contradictory in the remaining seven cases. Moreover, the single pure anti-idiotypic HAMA (B.1) and one of the anti-isotypes (A.7) are not detected by the combined IEMAs.

It is probably because of the increased analytical sensitivity, and maybe also, the more broad-spectrum specificity of the homologous Assay 4a that this assay showed positive results with seven sera from Patients 3, 22 and 28 (Group D.1-7, Table 2). These serum samples always remained negative with the OV-TL 3 based assays. Because the sera from these patients included their preinjection samples, the detected immunoresponses should be considered as pre-existing HAMA not related to OV-TL 3 or IgG_1 subclasss, but rather to other IgG subclasses. Thus, this preinjection, pre-existing HAMA may be considered a nonspecific response. Naturally occurring anti-mouse activity in blood was demonstrated in serum of more than 90% of healthy individuals (*3*) and may be a result of vaccination in the past, animal handling, dietary exposure, or cross-reactivity with rheumatoid factors. Furthermore, the suggestion of a nonspecific preinjection HAMA response is confirmed by negative scores with the modified Assay 4b (that includes the $F(ab')_2$ of OV-TL 3 as the capture antibody), as well as with all the OV-TL 3 related assays and the other general heterologous IgG_1-based Assay 4. Finally, the putative pure anti-Id HAMA (sample B.1) was neither reactive with the homologous Assay 4a, nor with the modified Assay 4b, probably due to the polyclonal signal antibody used in either IEMA not having a suitable epitope able to react with the anti-Id HAMA. This is irrespective of the reaction that should occur between the anti-Id HAMA and a suitable idiotope located within the hypervariable region of the OV-TL 3 Mab used as the capture antibody in Assay 4b.

In conclusion, the combined use of the developed OV-TL 3 related assays allows one to identify, and discriminate between anti-iso- and anti-idiotypic OV-TL 3 induced HAMA responses. Thus, it appears appropriate to use

these specific HAMA assays only after injection with OV-TL 3. The results of these assays may predict interference of HAMA, if any, with subsequent OV-TL 3 infusions. Also, the kinetic data of Figure 1 indicate that multiple time points, also beyond wk 6 postinjection, are needed in order to properly assess HAMA development, especially since the development of anti-Id HAMA has been reported to improve survival of ovarian cancer patients after immunoscintigraphy (*6,7*).

The low HAMA response with only 7 of the 33 patients studied may also indicate that the relatively short period of 6 wk postinjection is probably not long enough to detect all HAMA development in the patients studied. No other data is available on HAMA development with this Mab, but data on HAMA using other murine Mabs are not always consistent with our findings. This may be due to differences in immunogenicity, amount of protein used and immunocompetence of the patients studied.

The two more general HAMA assays have been shown to be less effective in detecting the OV-TL 3 induced HAMA (due to the combination of the two more general HAMA assays being required to detect almost all OV-TL 3 induced HAMA of Groups A and B, Table 2), despite the more diverse, broad-spectrum specificity (and sensitivity) of these assay systems. This may at least in part be explained by the inclusion in these assays of many different mono- or polyclonal reagent antibodies that are not always allotypically and isotypically matched to a particular HAMA, especially those developing after injection of OV-TL 3. However, the two more general HAMA assays tested appear to be useful for the identification of pre-existent HAMA and should therefore, be used prior to the first antibody injection.

Finally, it remains to be investigated whether the measurement of pre-existent HAMA is relevant for the assessment of the efficacy of the immunoscintigraphic treatment of patients, that is, by correlating how much and what type of HAMA interferes with the monitoring of tumor mass.

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