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Characterization of Human IgG Antimouse Antibody in Patients with B-Cell Malignancies[1]

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Abstract

Purpose: **Immunotherapeutic approaches to cancer offer an attractive adjunct to conventional modalities, although human antiglobulin responses can be an obstacle to repeated treatment. This study of a large number of patients with B-cell malignancies, over an extended period of time, characterized their human antimouse antibody (HAMA) seroconversion.**

Experimental Design: **A total of 617 samples from 112 subjects were analyzed for HAMA titers. Eighty-five patients with B-cell malignancies; 12 breast cancer patients, and 15 volunteers were titered for comparison. Fifty-six B-cell malignancy patients were titered for HAMA throughout Lym-1 radioimmunotherapy (RIT); 29 were titered after a single imaging dose of Lym-1 antibody.**

Results: **Baseline titers did not correlate with subsequent HAMA seroconversion against Lym-1. Only 1 of 29 (3%) of the patients developed HAMA after an imaging dose of Lym-1. Of the RIT trial group, 37 of 56 (66%) never developed HAMA above baseline despite multiple doses. Of those who did (19 of 56; or 34%), the HAMA responses fell into two categories. Thirteen responded rapidly (median of 31 days) and were termed "early responders," whereas 6, termed "late responders," had a median response time of 111 days. Early responders developed higher peak HAMA titers with fewer exposures to Lym-1 and took longer to return to baseline than did the late responders. The frequency of new antiglobulin seroconversion decreased as the number of exposures increased.**

Conclusions: **Seventy-seven percent of B-cell malignancy patients developed no response or a weak response after multiple doses of mouse Lym-1 antibody. Positive responders occurred in all histology types and fell into two categories differing in seroconversion time and titer, possibly indicative of the initial state of the immune system.**

Introduction

Immunotherapy and RIT[3] represent novel cancer treatments that have begun to fulfill their promise. An antiglobulin response to the antibodies used for these therapies can be an obstacle to repeated administrations of the antibody. Contrary to earlier expectations, anaphylactoid and adverse reactions have not proven to be significant problems (1). However, alterations of biodistribution and pharmacokinetic behavior of subsequently administered antibody because of complex formation can lead to less effective therapy. Thus far, immunotherapy and RIT have proven efficacious in the B-cell malignancies and repeated administrations have been feasible, in part, because of less likelihood of induction of an antiglobulin response in these malignancies. Despite humanization of the antibodies used for immunotherapy and RIT, HAMA responses to the residual mouse region continue to occur, albeit at a substantially reduced frequency and titer. In a trial of rituximab (Rituxan; IDEC Pharmaceuticals, San Diego, CA), immunotherapy that led to drug approval, a HAMA response rate of ~1% occurred in patients with low-grade, NHL after four weekly cycles of a chimerized anti-CD20 antibody (2). On the basis of remarkable results from the pivotal Phase III trials (3–5), one new drug application has been approved by the USFDA for the use of a radiolabeled antibody for RIT in low-grade and transformed NHL and another approval is pending. Both of these trials involved the use of radiolabeled mouse anti-CD20 antibodies. Although these trials involved a single therapeutic administration of the antibody, earlier trials involved multiple administrations. The frequencies of HAMA response to these and two other radiolabeled antibodies, anti-CD22 and Lym-1, used for RIT in patients with NHL of B-cell origin has been reported to be 1–42% (6–11), but the characteristics of the HAMA responses, the relationships to antibody exposures and amounts, and so on have not been reported in detail.

The purpose of this article is to better characterize the HAMA response of patients with B-cell malignancies (NHL and CLL) after multiple administrations of a mouse anti-B-cell HLA-DR antibody. A sensitive solid-phase ELISA was developed to quantify the HAMA titer at baseline and after each of multiple administrations of antibody. Baseline HAMA titers were evaluated for correlations with clinical parameters, including therapeutic response, and subsequent HAMA titers were

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[3] The abbreviations used are: RIT, radioimmunotherapy; HAMA, human antimouse antibody; NHL, non-Hodgkin's lymphoma; USFDA, United States Food and Drug Administration; CLL, chronic lymphocytic leukemia; LDH, serumlactic dehydrogenase; CV, coefficient of variation; MTD, maximum-tolerated dose.

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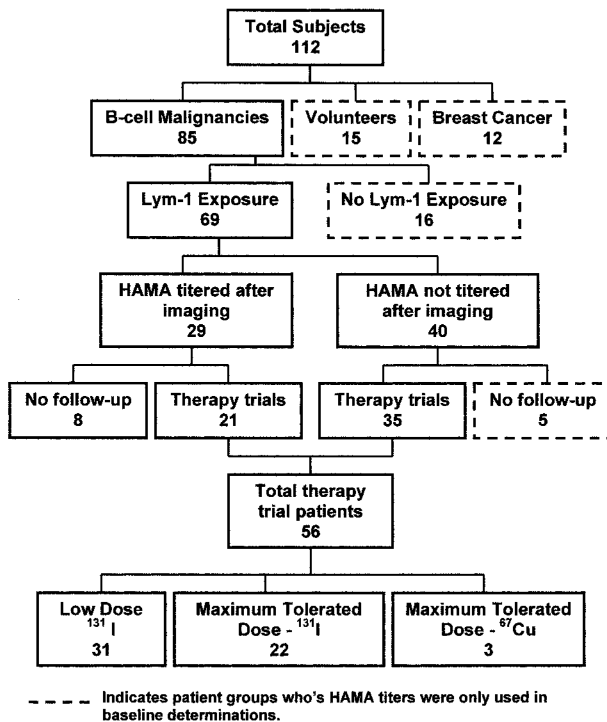

Fig. 1 Flow chart delineating the groups and subjects from whom serum samples were taken for the HAMA study. A total of 617 samples were assayed. Baseline titers were determined using 112 samples. A total of 505 samples were titered from B-cell malignancy patients after exposure to Lym-1.

Table 1 Characteristics of the patient group titered for HAMA throughout RIT treatments (n = 56)

	No. of patients	Percentage	Median	Range
Total	56	100		
Age (yr)			54	29–74
Male	33	59		
Female	23	41		
Histologic type				
NHL	51	91		
CLL	5	9		
Histologic grade				
Low[a]	18	32		
Intermediate[b]	30	54		
High	8	14		
Baseline HAMA (μg/ml)			0.3	BDL–4.5[c]

[a] The CLL patients are included in this group.
[b] Includes a patient with Richter's lymphomatous transformation of CLL.
[c] BDL, below detectable limits.

related to Lym-1 exposures and cumulative amounts (mg). Additionally, baseline HAMA titers in the patients with B-cell malignancies were compared with those of patients with breast cancer and volunteers, using the same assay.

Materials and Methods

Study Design and Population. Eighty-five patients with B-cell malignancies accessed Lym-1 RIT trial protocols and were analyzed for baseline HAMA and other parameters at the time using existing assay systems (9). Upon subsequent development of a more sensitive and accurate ELISA assay described herein all available samples, both baseline and posttreatment from these patients were retrospectively analyzed for HAMA. Samples from breast cancer patients and volunteer subjects were included in the study as controls for the analysis of baseline HAMA titers to allow comparisons of the test with those presenting with the targeted condition.

The majority of treated patients received an imaging dose followed within 1 week by a therapy dose of Lym-1. A dose was defined as any occasion on which Lym-1 was administered to a patient. Because two doses given at an interval of <7 days cannot be distinguished with regard to their ability to induce seroconversion (12), doses separated by <7 days were counted as 1 exposure.

A total of 617 serum samples from 85 patients with B-cell malignancies (76 NHLs, 9 CLL), 12 breast cancer patients, and 15 volunteers were quantified for HAMA (Fig. 1) using a capture ELISA assay. Baseline HAMA titers were assayed on 112 serum samples, and 505 samples were used to titer HAMA after exposure(s) to Lym-1 antibody. None of the patients with breast cancer or the volunteers had known prior exposure to Lym-1 or any other monoclonal antibody. All patients with B-cell malignancies were included in the baseline HAMA evaluation. Fifty-six of these patients had subsequent exposure to Lym-1 antibody as participants in one of several RIT trials, and an additional 13 had exposure to imaging doses of Lym-1 only. These exposures occurred after the baseline HAMA assay that was used to determine their eligibility to participate in the RIT trials that involved administration of therapeutic amounts of radioisotope (^{131}I or ^{67}Cu) attached to Lym-1 antibody (14–100 mCi/m^2 given at intervals of 2–6 weeks; Refs. 8, 13). The HAMA profile of the 56 treated patients who were given multiple doses of Lym-1 was assessed. Twenty-nine of the patients had serum samples available that had been drawn after their imaging study to allow assessment of HAMA after a single imaging dose of Lym-1. The characteristics of the B-cell malignancy patient populations assessed for HAMA either after sequential RIT trial exposures (Table 1) or after one imaging exposure (Table 2) have been summarized.

All imaged and treated patients were advised of the investigational nature of the studies and signed consent forms approved by the University of California Davis Human Subjects and Radiation Use Committees under Investigational New Drug authorizations from the USFDA.

As per protocol requirements, patient's serum, except where noted, was obtained before Lym-1 administration (baseline titer), 2–6 weeks after each Lym-1 exposure (before subsequent Lym-1 exposure), as well as 6 weeks and 6 months after completion of or removal from a trial. Twenty-one patients were followed for ≥6 months. Additional samples were obtained to follow HAMA-positive patients. HAMA titers were examined using a range of metrics (numbers of doses, exposures, and milligram amounts of Lym-1).

Patients entering a RIT trial had a complete blood count and full chemistry panel done within 7 days before their first

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Table 2 Characteristics of the patient group titered for HAMA after a single imaging dose of Lym-1 ($n = 29$)

	No. of patients	Percentage	Median	Range
Total	29	100		
Age (yr)			53	29–74
Male	16	55		
Female	13	45		
Histologic type				
NHL	26	90		
CLL	3	10		
Histologic grade				
Low[a]	7	24		
Intermediate[b]	15	52		
High	7	24		
Baseline HAMA (μg/ml)			0.43	BDL–4.2

[a] The CLL patients are included in this group.
[b] Includes a patient with Richter's lymphomatous transformation of CLL.
[c] BDL, below detectable limits.

exposure to Lym-1. Those values were used to correlate information on serum protein, globulin, LDH, WBC, and lymphocyte counts.

Pharmaceuticals. Lym-1, a mouse IgG2a monoclonal antibody with high affinity for a M_r 31,000–35,000 antigen on the surface of malignant B cells (14, 15), was produced in ascites in our laboratory or supplied by Damon Biotech, Inc. (Needham Heights, MA) or Techniclone, Inc. (Tustin, CA). Quality assurance of Lym-1 lots showed >95% monomeric IgG by PAGE. Lym-1 antibody preparations met USFDA mouse antibody production guidelines for murine viral, *Mycoplasma*, fungal and bacterial contamination, endotoxin, pyrogen, DNA content, and general safety testing in animals.

Lym-1 was radiolabeled with: (*a*) ^{131}I or ^{123}I using chloramine T; (*b*) ^{111}In using the bifunctional chelator *p*-isothiocyanatobenzyl-EDTA or the macrocyclic bifunctional chelator 2-[p-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclododecane-*N,N′N′′,N‴*-tetraacetic acid; or (*c*) ^{67}Cu using the macrocyclic bifunctional chelator 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N,N′,N′′,N‴*-tetraacetic acid as described previously (13, 16, 17). Quality assurance of the radiopharmaceuticals by size exclusion high-performance liquid chromatography showed >90% of the radioactivity was associated with monomeric IgG, and immunoreactivity was >75% (18). All preparations were sterile and pyrogen free.

Human IgG Antimouse Antibody (HAMA) Assay. HAMA titers were quantified in an ELISA format using 96-well plates (Pro-Bind; Becton Dickinson, Lincoln Park, NJ). Briefly, the plates were coated with 2 μg (100 μl)/well of Lym-1 at pH 8.9 for 1 h at 37°C. Nonspecific binding sites were blocked with 5% PBS/BSA for 30 min. After this and subsequent incubations, wells were washed three times with PBS containing 0.1% Tween 20. PBS/BSA was used as the sample blank to determine background absorbance. Replicate (50 μl) serum samples in PBS/BSA (undiluted, 1:10 and 1:100) were incubated for 1 h at 37°C. Bound human IgG was detected using biotinylated, Fc-specific goat (Amersham, Arlington Heights, IL) or mouse (Sigma, St. Louis, MO) antihuman IgG followed by streptavidin-horseradish peroxidase (Amersham) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma). After 10 min at room temperature, the reaction was stopped with 50 μl of 10% SDS. The absorbance of each well was read immediately at 410 nm in an ELISA reader (Dynatech MR-300, Chantilly, VA).

A reference standard was generated from plasmaphoresis material obtained from a NHL patient that had developed HAMA after 2 exposures and a total of 183 mg of Lym-1 (19). The serum HAMA titer was 61 μg/ml before plasmaphoresis, and the HAMA titer of the plasmaphoresis material was 7.2 μg/ml. The amount of HAMA in the reference standard was determined by a direct method (12, 19). Briefly, Lym-1, trace labeled with ^{125}I using the chloramine T method, was incubated with serum. The mixture was then subjected to size exclusion high-performance liquid chromatography analysis. Percentage of radioactivity from the fractions was calculated to assure 100% recovery. Quantitation of HAMA in the serum was then possible using the total radioactivity eluted in fractions corresponding to a molecular weight of M_r 300,000. The percentage of radioactivity in these fractions multiplied by the amount of Lym-1 added yielded the amount of HAMA, assuming a 1:1 ratio of ^{125}I-Lym-1 to HAMA in the M_r 300,000 peak. Serial 2-fold dilutions of a serum sample containing a known amount (7.2 μg/ml) of HAMA were used to generate the reference standard curve for quantifying HAMA in each ELISA. The reference standard curve in this assay was reproducible with a linear portion between 0.00045 and 0.0072 μg or the equivalent of 0.009–0.144 μg/ml of IgG HAMA in serum. The average slope of the linear portion of the curves from 10 assays was 1.40 ± 0.18 (CV = 12.6%). The lowest detectable limit for HAMA was 0.00025 μg in a 50-μl sample or the equivalent of 0.005 μg/ml (5 ng/ml) IgG HAMA in serum. The accuracy as tested by directly coating human IgG onto the plate at various concentrations was confirmed over the same linear range as the reference standard.

The HAMA titer was defined as the amount in the least dilute sample that fell within the linear portion of the standard curve. A positive HAMA titer was defined as a value >5 μg/ml or 4 SDs above the mean of the baseline titer for the volunteer group. Assuming that the HAMA titers were normally distributed, the selection of a value 4 SDs above the sample mean provided >95% confidence that the chance of a seronegative individual having a value > 5 μg/ml was <1%. This value was larger than that seen for any of the 15 volunteers and 56 RIT trial patients at baseline. The reproducibility of this HAMA assay as demonstrated by repeated tests on 9 patient samples with HAMA titers from 0.2–50 μg/ml gave a CV < 25%, which is similar to that reported for other HAMA assays (12, 20, 21). A dilution of 1:100 of the reference standard (from the linear portion of each standard curve) also gave a CV < 25% over a 2-year period.

Statistical Methods. The nonparametric Kruskall Wallis Rank-Sum test (22) was used to test for differences between data from independent groups (*e.g.,* differences between baseline values or clinical parameters for HAMA responders *versus* nonresponders). Where multiple groups were compared (*e.g.,* volunteers *versus* lymphoma patients *versus* breast cancer patients), an initial overall comparison was done. Only if the results were statistically significant were the paired comparisons

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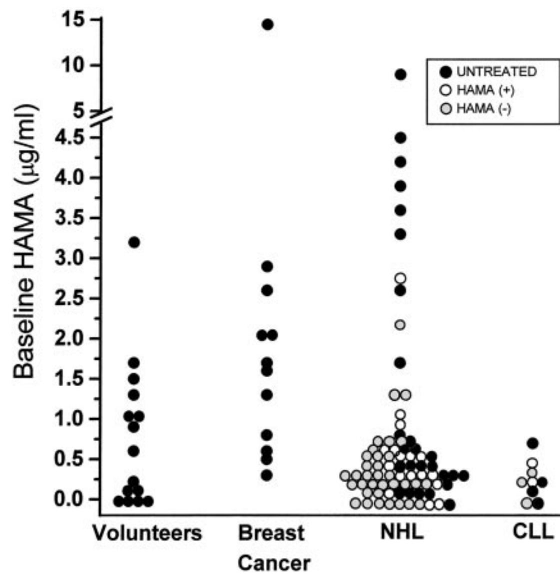

Fig. 2 Baseline HAMA titers in patients with B-cell malignancies and controls (volunteers and patients with breast cancer). Baseline HAMA titers for the volunteers and B-cell malignancy groups were not significantly different from each other, but both were lower than the breast cancer group ($P < 0.05$).

tested. This approach was used to avoid the likelihood of false positives because of multiple comparisons. To test for association among continuous variables (*e.g.,* associations between baseline HAMA titers and clinical parameters within the treated B-cell malignancy patient group), Kendall τ (22) was used. Fisher's exact test (23) was used for analyses of dichotomous responses between two groups (*e.g.* proportion females). Nonparametric exact tests were used in all of the analyses (StatXact-Turbo; Cytel Corp.) because of the nonnormality of the data in many cases and because of the small sample size of some cohorts. Significance was assessed using $P < 0.05$ as the critical value in all analyses.

Results

Baseline HAMA Titers. Baseline HAMA titers have been quantified for 112 individuals (Fig. 2). The volunteers had a median baseline titer of 0.6 μg/ml (range <0.005–3.2 μg/ml). The total B-cell malignancy population (85 patients) had a median baseline titer of 0.3 μg/ml (range, <0.005–8.8 μg/ml). For comparison, 12 breast cancer patients, a group with a high HAMA response rate (24, 25) had a median baseline titer of 1.6 μg/ml (range, 0.3–14.9 μg/ml). Comparison of baseline HAMA titers of the three groups was statistically significant ($P = 0.0005$). Additional comparisons between the individual groups demonstrated no significant difference between the volunteer group and the B-cell malignancy group at baseline ($P = 0.334$) but both were lower than the breast cancer group ($P < 0.05$). There were 2 (2%) of 112 individuals (1 breast cancer and 1 B-cell malignancy patient) with baseline HAMA titer > 5 μg/ml serum and no known prior exposure to mouse antibody.

Of the 56 RIT patients, all of whom had baseline HAMA titers < 3 μg/ml, there was no association of baseline titer with

Table 3 Comparison of patients who developed positive HAMA titers with those who did not

	HAMA (+) $n = 19$	HAMA (−) $n = 37$
Age (yrs)	60 (37–71)[a]	52 (29–74)[a]
Male	13/33	20/33
Female	6/23	17/23
Histologic grade		
Low	7/18	11/18
Intermediate	9/30	21/30
High	3/8	5/8
Histologic type		
NHL DLC[b]	9/27	18/27
NHL follicular	5/14	9/14
CLL	2/5	3/5
Other	3/10	7/10
Trial		
Low-dose-^{131}I	10/31	21/31
MTD-^{131}I	8/22	14/22
MTD-^{67}Cu	1/3	2/3

[a] Median (range).
[b] DLC, diffuse large cell.

age, sex, histological grade or type, serum protein, globulin, LDH, WBC, or lymphocyte count (all Ps > 0.1). Baseline HAMA titers also did not correlate with subsequent HAMA responses to Lym-1 therapy ($P > 0.1$). There was no difference in baseline HAMA titers between patients that developed a HAMA response (median, 0.4 μg/ml) and those without a HAMA response (median, 0.2 μg/ml; $P = 0.0913$).

HAMA Responses. There were no differences in the frequency of HAMA response based on therapy trial, age, sex, histological grade, or type (Table 3). Nineteen of the 56 (34%) RIT patients seroconverted against Lym-1 during therapy; 10 of 31 (32%) ^{131}I-Lym-1 low-dose patients, 8 of 22 (36%) ^{131}I-Lym-1 MTD patients, and 1 of 3 (33%) ^{67}Cu-2IT-BAT-Lym-1 MTD patients. Seventeen of 51 (33%) NHL patients and 2 of 5 (40%) CLL patients became seropositive. Seven of 18 (39%) low-grade (including the CLL patients), 9 of 30 (30%) intermediate grade, and 3 of 8 (38%) high-grade lymphoma patients seroconverted after therapy. Nine of 27 (33%) of patients with diffuse large cell types of NHL, the prevalent histological type, and 10 of 29 (34%) of the patients with other histological types became seropositive. Although the number of each type of histology was too small for statistical analysis, antiglobulin responses were seen in almost all types of histology represented in this population of B-cell malignancies.

Only 1 of 29 (3%) of the patients who were evaluated after receiving a single imaging dose of Lym-1 developed a positive HAMA titer. This patient became seropositive by 9 days after a single dose of 70 mg of ^{67}Cu-2IT-BAT-Lym-1. The median amount of Lym-1 received, in a single dose, by the 29 patients was 20.4 mg (range, 0.22–70 mg). The median time for HAMA assessment after the dose was 26 days (range, 8–93 days). Twenty-one of these patients were later entered into a Lym-1 RIT trial.

The characteristics of the HAMA responses of the 19 RIT patients who became seropositive are summarized in Table 4. The median amount of Lym-1 administered to the time of seroconversion was 113 mg (range, 24–216 mg), compared with a median of 143 mg of Lym-1 (range, 8–1045 mg) administered

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Table 4 Characteristics of the HAMA(+) population

Patient	Trial design	HAMA (μg/ml)		Total Lym-1 (mg)	Total mCi	Time/total amounts to positive HAMA			Peak HAMA
		Baseline	Peak			Days from first dose	Lym-1 (mg)	mCi	Days from first dose
1	LD[a]	0.44	1802	196	243	31	113	120	73
2	LD	0.03	10.3	179	389	99	148	291	119
3	LD	0.97	13.1	125	258	144	125	258	151
4	LD	0.44	1444	298	241	30	157	64	184
5	LD	0.32	50	164	157	38	154	108	51
6	LD	0.55	1313	47	195	51	24	75	112
7	LD	0.59	87.6	310	269	45	216	96	136
8	LD	0.24	118	39	111	111	27	67	111
9	LD	0.22	6.6	247	501	296	164	382	332
10	LD	0.54	42	24	88	39	24	88	39
11	MTD	0.25	26.7	104	208	23	53	109	28
12	MTD	0.09	9.9	61	175	28	61	175	28
13	MTD	0.71	6.7	214	517	112	214	517	112
14	MTD	BDL	148	162	448	51	97	305	86
15	MTD	0.47	163	68	225	23	68	225	67
16	MTD	2.66	1570	126	316	21	60	166	51
17	MTD	0.26	87.6	52	102	24	52	102	24
18	MTD	2.20	1048	118	358	72	45	206	114
19	CuRx	0.27	31	134	131	79	134	131	79
Median		0.44[b]	87.6	126[c]	241[d]	45	113	131	86

[a] LD, low-dose ^{131}I-Lym-1 trial; MTD, ^{131}I-Lym-1 MTD trial; CuRx, ^{67}Cu-2IT-BAT-Lym-1 trial; BDL, below detectable limits.
[b] Compared with median baseline titer of 0.23 mg/ml for the HAMA nonresponder population.
[c] Compared with median total Lym-1 of 143 mg administered to the HAMA nonresponder population.
[d] Compared with median total mCi of 301 administered to the HAMA nonresponder population.

to the 37 RIT patients who remained seronegative. No patient receiving <24 mg of Lym-1 developed HAMA, and patients receiving as much as 1045 mg of Lym-1 in as many as 21 doses or 19 exposures remained seronegative. The fewest doses and exposures to elicit HAMA in the RIT group were 2 and 1, respectively. The average amount of radioactivity received was similar for both HAMA-seropositive and -seronegative groups (Table 4).

Positive HAMA seroconversion in the RIT group was first detected at a median of 45 days (range, 21–296 days) after the first dose of Lym-1. However, a more meaningful comparison exists when the HAMA responders are grouped as follows. Thirteen of the 19 patients (68%) that seroconverted did so within 72 days (median of 31 days) after their first dose of Lym-1. Six patients became seropositive at ≥77 days (median of 111 days) after their initial dose of Lym-1. We have classified these two groups as early and late responders based on elapsed time from first dose to a documented seroconversion. Characteristics of the early and late responders are illustrated in Fig. 3. Early responders received a median of 1 exposure (range, 1–3 exposures; Fig. 3*A*) or 2 doses (range, 2–4 doses) of Lym-1 before seroconversion. In contrast, the late response group received a median of 4 exposures (range, 2–7 exposures) or 5 doses (range, 2–9 doses) before seroconversion. The amount of Lym-1 administered before development of a HAMA response (Fig. 3*B*) did not differ for early and late responders. Early responders, however, developed significantly higher peak HAMA titers than late responders ($P < 0.01$; Fig. 3*C*). Among the 13 early responders, 5 (38%) developed peak HAMA titers > 1000 μg/ml serum. Three of these 5 patients survived ≥5 years after their initial Lym-1 exposure.

Twelve (63%) of the 19 patients who seroconverted subsequently received additional therapeutic doses of Lym-1. These patients were given doses of unconjugated Lym-1 to complex the circulating HAMA before RIT (19). There were no anaphylactoid responses in any of these patients. The development of HAMA titers in 6 (11%) of the 56 treated patients interrupted additional Lym-1 therapy.

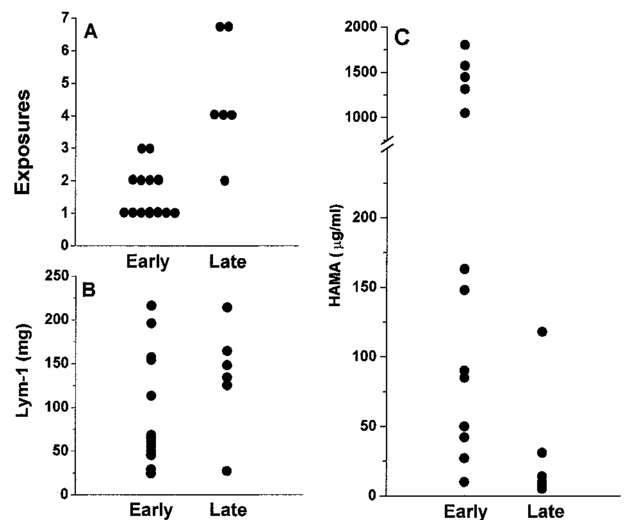

Fig. 3 Characteristics of the HAMA response to Lym-1 of patients with B-cell malignancies. Early responders (median conversion time, 31 days) received fewer exposures than did the late responders (median, 111 days; *A*). Although total amounts of Lym-1 administered within each group did not differ (*B*), the highest peak HAMA titers occurred in the early responders with three of these patients having prolonged survival times. Early responders developed significantly higher HAMA titers than late responders ($P < 0.01$; *C*).

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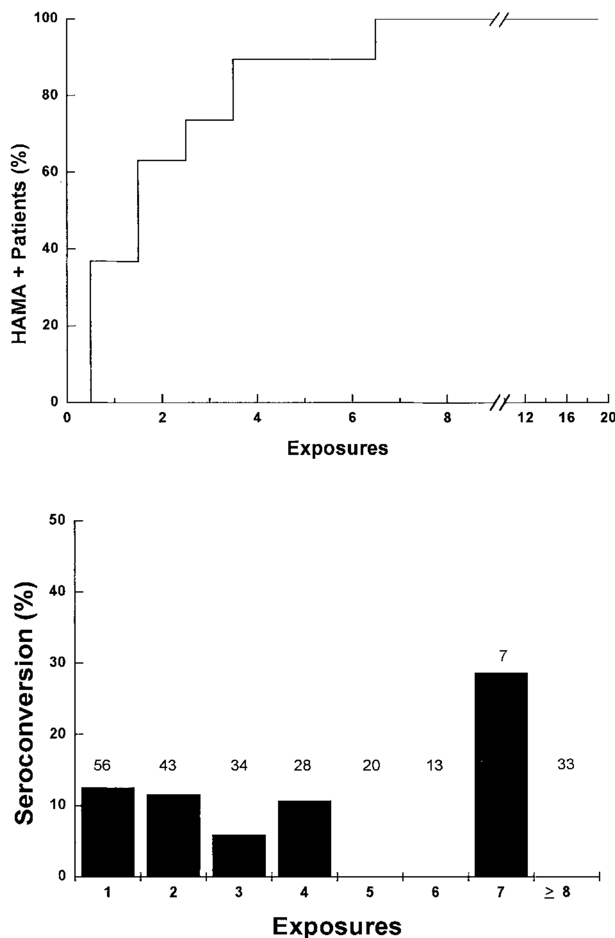

Fig. 4 Relationship of seroconversion to exposures. The percentage of 19 patients that had converted relative to number of exposures (*top*). Likelihood of seroconversion relative to the total number of Lym-1 exposures (*bottom*). Two patients weakly seroconverted after their seventh exposure. Five patients had 33 exposures beyond 7 and 1 of those patients had 19 total exposures, but all 5 remained seronegative.

Four patients who developed HAMA have been followed long enough to demonstrate a return to normal baseline titers. Two of these patients (nos. 7 and 15 from Table 4) were early responders and took 11 and 22 months, respectively, from their last dose of Lym-1 for their HAMA to return to normal. The other 2 patients were late responders (nos. 3 and 19) and their HAMA took only 2 and 5 months, respectively, to return to normal. Early responding patient nos. 1, 4, 14, 16, and 18 were followed 400, 3225, 293, 1519, and 1281 days, respectively, after their last Lym-1 exposures and were still positive for HAMA.

The 4 longest surviving patients were all early HAMA responders. Two had survival times of 5 years, 1 survived 11 years, and 1 patient is still alive 12 years after initiation of RIT.

The likelihood of developing a HAMA response in the RIT patient groups is illustrated in Fig. 4. Of those patients who seroconverted (*top panel*), the majority (>60%) did so after 1–2 exposures. Patients who failed to seroconvert after two exposures to Lym-1 had a decreased likelihood of subsequent seroconversion with continued exposures. Five patients received 8–19 exposures and all remained seronegative.

Discussion

Patients with B-cell malignancies such as NHL and CLL have been documented to be immunodeficient (26). Although immunodeficiency varies in these patients, some fail to respond to any of the traditional tests for immunocompetence and as many as 80% are unresponsive to any single test for immunocompetence. Interestingly, patients with T-cell malignancies readily mount HAMA in response to exposure to mouse antibodies (27). About 48–75% of patients with solid tumors develop HAMA after a single dose of mouse antibody (28, 29). In contrast, 1–42% of patients with B-cell NHL develop HAMA, even if given multiple doses of mouse antibodies in large amounts (6–9, 11, 30). Despite a number of excellent publications on the antiglobulin response to mouse, chimerized, and humanized mouse antibodies (20, 31, 32), the baseline titers and HAMA responses to multiple doses of mouse antibody given over time have not been characterized in a population of patients with B-cell malignancies, nor have they been correlated with demographic and clinical characteristics. Additionally, baseline HAMA titers have not been previously analyzed for their predictive value for HAMA responsiveness.

We developed and carefully characterized a quantitative HAMA assay for these purposes. The sensitivity of this capture ELISA assay was 0.00025 μg in a 50-μl sample or 0.005 μg/ml (5 ng/ml) serum. The Lym-1 antibody administered to the patients was also used as the capture antibody, as recommended by the HAMA international survey group (21). In this way, all types of HAMA (idiotype, isotype, species) present in the sample were detected by our assay. Our HAMA assay method met the requirements preferred by the international HAMA survey group. For example, the assay used an antihuman IgG detection antibody to detect lower affinity antibodies because both antigen binding arms of the HAMA are available for binding to the capture antibody. The international survey found qualitative agreement but significant differences in quantitative results using different assay methods. Our data seem likely to be accurate because they were verified by titration of serum HAMA against Lym-1 *in vitro* and *in vivo* (19).[4] In any event, the data are precise, and the comparisons valid because the patients served as their own control.

Using this assay, the frequency of HAMA responses was 1 of 29 (3.4%) in patients after a single imaging dose of mouse Lym-1 antibody and ~35% for three different groups of patients treated with multiple therapy doses of ^{131}I- or ^{67}Cu-labeled Lym-1. In the case of imaging, a single dose of a few milligrams, or fractions thereof, were given, except for the patient that developed a HAMA. In this instance, 70 mg of Lym-1, necessitated by the specific activity of the ^{67}Cu supply, were given. In the case of therapy, larger amounts of antibody were required to deliver the desired therapeutic amounts of ^{131}I- or ^{67}Cu-Lym-1. In addition to the previously cited evidence for the

[4] Unpublished data.

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immunodeficient state of these patients, their baseline HAMA titers were significantly lower than those of patients with breast cancer ($P < 0.01$) when tested against Lym-1, using the same assay. Baseline HAMA titers of the patients with B-cell malignancies clustered <1 μg/ml, but a few of the patients with NHL had higher HAMA titers. Because protocol requirements precluded entry of patients into the therapy phase of the trials at HAMA titers > 5 μg/ml, the predictive value for subsequent HAMA response of higher baseline HAMA titers cannot be commented upon. However, there was no significant degree of correlation between baseline HAMA titer and demographic or clinical parameters, nor was there predictive value between baseline HAMA titer and HAMA response to Lym-1 exposure for those patients with baseline HAMA titers < 5 μg/ml.

Because the therapy trials were designed for administration of multiple doses of Lym-1, they provided an opportunity to characterize HAMA responses over time and their implications in a population of patients with advanced NHL and CLL. There was evidence for immunodeficiency in the patient population. Some patients seemed unable to generate a HAMA even when given a great many doses of Lym-1. HAMA responses fell into three rather distinct groups: negative, early, and late responders. Thirty-seven of 56 (66%) patients never generated a HAMA response to Lym-1 therapy, even after many doses and large amounts of Lym-1; 1 of these patients remained HAMA negative after having received 21 doses (19 exposures) and 1045 mg of Lym-1. At the other extreme, there was a group of patients (13 of 56; 23%) that we categorized as early responders because they developed high HAMA titers soon after receipt of 1 or 2 doses of Lym-1. HAMA responses in these patients seemed similar to that expected for immunocompetent individuals. Finally, there was a group of patients (6 of 56; 11%) that required many doses of Lym-1 before they developed a HAMA response of low titer.

Baseline HAMA did not correlate with demographic parameters or clinical parameters such as serum LDH (tumor burden), blood counts (lymphocytes), serum proteins, and so forth. Despite this, HAMA responsiveness did seem to infer the immune status of the patient, with important clinical implications, as will be commented upon later. In contrast to conventional immunological wisdom, the frequency of new HAMA seroconversions in these patients actually decreased with increasing dose number (Fig. 4). Additionally, HAMA responsiveness did not correlate with the amount of administered Lym-1 (Fig. 3). Although reported for heterogeneous patient populations and chimerized mouse antibodies, Kuus-Reichel *et al.* (31) found little relationship between total dose of antibody and HAMA responses (<1–200 mg) but did observe some increase in HAMA responses in patients that received 2–5 doses when compared with a single dose. HAMA titers were higher in the group that we defined as early responders, although they received fewer doses/exposures and equivalent amounts of Lym-1 than the late responders. Furthermore, late responders returned to normal HAMA titers rapidly, whereas the early responders required a longer interval of time to return to a normal HAMA titer; several did not reach baseline during follow-up of as long as 11 years. This pattern of responsiveness is suggestive of dependence upon the initial immune status of the patient.

Patients with low HAMA titers could be, and in some instances were, treated with additional Lym-1 therapy using amounts of Lym-1 sufficient to complex the circulating HAMA so that the radiolabeled Lym-1 could target the malignant cells. This strategy proved effective as judged by blood clearance, tumor targeting, and additional tumor regression. Importantly, anaphylactoid reactions did not occur, and adverse events were not greater than those for HAMA-negative patients (data not shown). Of greater importance, the survivals of patients that developed a HAMA response to Lym-1 therapy tended to be longer than those of the patients that remained HAMA negative. Survivals may reflect greater initial levels of immunocompetence among the patients. HAMA was shown to include generation of anti-idiotypic antibodies that recognized mirror images of the antigen on the malignant lymphocytes in some of these patients. In turn, some of these patients generated antibody titers against their anti-idiotypic antibodies and, therefore, potentially, the antigens on their malignant lymphocytes (data not shown). These idiotypic cascades in response to mouse antibodies have been shown to have survival advantage (33–35).

As indicated earlier, new drug applications that have been approved (or are pending approval) by the USFDA seek to use two drugs, ^{90}Y-labeled ibritumomab (Zevalin; IDEC Pharmaceuticals) and ^{131}I-labeled tositumomab (Bexxar; Corixa-Coulter Pharmaceuticals, Palo Alto, CA). Although the pivotal Phase III trials used single doses to achieve remarkable response rates for Zevalin and Bexxar in low-grade NHL (11, 36, 37), the Phase I-II trials of these antibodies often used multiple doses (6, 38–41). There are both similarities and differences between these trials and those reported herein for Lym-1 therapy. Large amounts of anti-CD20 antibody, chimerized mouse (Rituxan) in the case of Zevalin and mouse in the case of Bexxar, were given before administration of the radiolabeled mouse anti-CD20 antibody, Zevalin or Bexxar, respectively, to improve targeting of malignant tissue. Depletion or substantial decrease in circulating B cells occurred in response to these biologically active antibodies. Although circulating lymphocytes are decreased by the radiation from Lym-1 RIT, Lym-1 antibody is given in much smaller amounts to target malignant tissue and has little depleting effect on normal lymphocytes. Despite these differences, the frequency of HAMA that we observed in response to Lym-1 therapy was similar to that reported in the Phase I–II trials of Bexxar but greater than that reported for Zevalin (11, 40, 41). The difference for Zevalin may relate to greater depletion of normal B lymphocytes because of the chimeric nature of the rituximab preload. Because detailed information has not been reported for the anti-CD20 antibodies, we can only speculate on the exact relationship of our Lym-1 data to RIT with these other antibodies. However, the demographic observations for Lym-1 therapy seem relevant to Bexxar when approved because multiple doses of this mouse antibody is likely to be administered sometimes to complete or solidify responses and to treat relapses.

In summary, the immunodeficient state of patients with NHL and CLL has been further corroborated. HAMA response to multiple doses of a mouse antibody, Lym-1, given over time to these patients has been characterized, with negative responders, early responders in high titer, and late responders in low titer regardless of the amounts of administered Lym-1. When

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patient baseline HAMA titers were less than the RIT trial eligibility criterion of 5 μg/ml, demographic and clinical data failed to predict likelihood of HAMA response to Lym-1 therapy. Effective Lym-1 therapy was continued in some of the HAMA responders with evidence for benefit and without evident increase in risk. HAMA responsiveness seemed to correlate with increased survival. This phenomenon is under further investigation in this laboratory.

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