Immunogenicity of the Hu14.18-IL2 Immunocytokine Molecule in Adults With Melanoma and Children With Neuroblastoma

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Abstract

Purpose: Immunocytokine (IC) hu14.18-IL2 is a fusion protein of humanized antisialosialoganglioside (GD2) antibody (hu14.18) and interleukin (IL)-2. Sixty-one melanoma and neuroblastoma patients received IC in phase I/ Ib studies. Patient sera were examined in ELISA to determine if an anti-IC antibody response occurred during treatment.

Experimental Design: Serum was assayed for anti-idiotypic antibody (anti-id Ab) based on ability to bridge biotinylated hu14.18 to plate-bound hu14.18 and ability to inhibit binding of hu14.18 to GD2 antigen and/or murine anti-idiotypic antibody. ELISA was also used to detect antibodies to the Fc-IL2 end of hu14.18-IL2.

Results: Thirty-two patients (52%) developed an anti-idiotypic antibody response (absorbance, >0.7) in the bridge ELISA. Twelve patients (20%) had an intermediate response, whereas 17 patients (28%) were negative (absorbance, <0.3). The development of antibody to hu14.18-IL2 detected in the bridge ELISA was not related to the dose of hu14.18-IL2. Twenty of 33 adult patients (61%) demonstrated an anti-idiotypic antibody response based on binding inhibition ELISA. The anti-idiotypic response was inversely correlated (P < 0.002) with IC measured during the second course of treatment, indicating that development of anti-idiotypic antibodies interfered with detection of circulating hu14.18-IL2. All patients developed some inhibitory activity in the binding inhibition assay designed to detect antibodies to the Fc-IL2 region of the IC. There was a positive correlation between the peak serum level of IC in course 1 and the anti-Fc-IL2 response.

Conclusions: Patients treated with hu14.18-IL2 developed anti-idiotypic antibodies and anti-Fc-IL2 antibodies. No association was seen between development of anti-IC antibodies and clinical toxicity.

In an effort to improve antitumor effects with interleukin 2 (IL-2; ref. 1) or monoclonal antibody (mAb; ref. 2) alone, or combined treatment with the individual components (3–7), an immunocytokine (IC; refs. 8, 9), which contains the tumor reactive 14.18 mAb linked to IL-2 at the carboxy terminus of each IgG1 heavy chain, was created. The proposed mechanism of action is localization to tumor via recognition of tumor-associated GD2 disialoganglioside (10–13). Localization of IC facilitates activation of natural killer cells through Fc and IL-2 receptors (14) and activation of T cells through IL-2 receptors (15). Natural killer cells mediate cytolytic activity via antibody-dependent cellular cytotoxicity and non–MHC-restricted cytotoxicity (9). In some preclinical models, tumor antigen–specific T-cell memory is also induced (15, 16). Clinical reports for separate phase I studies treating melanoma (MEL) and neuroblastoma (NBL) patients with this IC were recently published (14, 17). The present study was designed to determine if patients receiving the IC developed an immune response to the IC. We monitored patients for development of antibody to the IC. Adult MEL patients with responding or stable disease were eligible to receive a second course of IC (14). Pediatric NBL patients with stable or responding disease were eligible to receive up to four or six courses of IC, respectively (17). We established ELISAs to detect antibodies specific for the two separate functional ends of the IC. Antibodies against the idiotypic determinant (18) and against the carboxy terminus of the IgG heavy chain where IL-2 is linked (Fc-IL2 end) were detected. These antibodies could potentially interfere with the proposed functions of the IC. An anti-idiotypic antibody might...
prevent the IC from targeting to tumor (18). An antibody against the Fc-IL2 end of the IC (anti–Fc-IL2) might interfere with immune activation facilitated through IL-2. We report here on the occurrence, frequency, and potential immunologic effects of the antibody response to hu14.18-IL2.

Materials and Methods

**Hu14.18-IL2.** IC (EMD 273063) was provided by EMD Pharmaceuticals, Inc., (now EMD Serono, Inc.). One milligram of IC contains 3 × 10^11 IU of IL-2 (19) and 0.8 mg of the hu14.18 antibody.

**Study design.** These phase I trials were nonrandomized dose escalation studies. Initial clinical and immunologic results were previously reported (14, 17). Briefly, hu14.18-IL2 was given as a 4-h i.v. infusion on days 1, 2, and 3 of each 28-d treatment course. Adult patients received up to two courses and pediatric patients received up to six courses of IC. Unless otherwise indicated, serum samples were taken with morning blood draws, before administration of IC. The day and course for blood samples are identified as follows: C1D1, course 1, day 1; C3D8, course 3, day 8. Peak IC serum levels were determined from blood obtained within 1/2 h of completing the IC infusion.

**Cell lines.** M21 (CD2–positive melanoma; refs. 14, 17) and IL-2 receptor–positive RL-12 (subline of NKL-human leukemia obtained from Dr. Paul Leibson of the Mayo Clinic, Rochester MN; ref. 20) were maintained as previously described.

**ELISAs**

**SIL-2Ra.** SIL-2Ra was measured by (Immunotech) ELISA kit.

**Detection of IC.** Measurement of IC in patients’ sera by ELISA was done as previously described (18, 21, 22).

**Detection of anti-IC antibodies.** The humanized 14.18-IL2 has two types of immunogenic epitopes recognized as foreign by some patients. The anti-GD2 idiotypic determinant and the determinants created where IL-2 is directly linked to the carbohydrate terminus of IgC heavy chains, called Fc-IL2. We have created ELISAs that allow the detection of antibodies to these determinants (Fig. 1).

- **Anti-Id-Bridge to detect anti-14.18 Idiotypic antibody is similar to our previously published “bridging” method detecting anti-14.18 chimeric antibody (Fig. 1A; ref. 23). Serum samples obtained before therapy from all patients were negative in this assay [adsorbance (OD), <0.3].**
- **Anti–IC-binding inhibition (GD2) for detection of anti-IC antibodies using GD2-coated plates (Fig. 1B; ref. 21). Hu14.18-IL2 binds to GD2 and is detected using biotinylated anti-IL2 antibodies. When patient serum containing anti-idiotypic antibody is combined with IC before adding to GD2-coated plates, the anti-idiotypic antibody inhibits the 14.18 antibody component of the IC from binding to GD2. In addition, when patient serum contains antibodies specific for the Fc-IL2 determinant, this antibody binds to the IC and inhibits the biotinylated anti-IL2 antibody from detecting the IC. Thus, this ELISA detects anti-IC antibodies against both idiotypic and Fc-IL2 determinants. Briefly, purified disialoganglioside GD2 (Advanced ImmunoChemical, Inc.) suspended in 100% ethanol was absorbed to 96-well flat-bottom polystyrene plates (Falcon #3915; Fisher Scientific) at a concentration of 250 pmol/L. Hu14.18-IL2 was diluted to 250 ng/mL and further diluted 1:2 with patient serum. This mixture was incubated at room temperature for 1/2 h and 100 μL per well added to duplicate wells and incubated for 2 h at 37°C. Hu14.18-IL2 bound to GD2 was detected using biotinylated goat-anti-human-IL2-antibody (8.33 ng/mL) and R&D Systems, followed by ExtrAvidin–horseradish peroxidase (HRP) conjugate (2 μg/mL; SIGMA-Aldrich Chemicals) and addition of TMB substrate (DARO Chemicals). The reaction was stopped using 50 μL per well of 2N H2SO4. The absorbance (OD OD0 to ODw) was used to determine the amount of hu14.18-IL2 detected interpolating to a standard curve. The % inhibition was then calculated as follows:

\[
\%\text{ inhibition} = \frac{OD_{w} - OD_{0}}{OD_{0}} \times 100
\]

The specificity of the anti-IC antibodies detected in the binding inhibition assays was assured by comparing IC detected using pretreatment serum to IC detected using serum obtained at times following treatment.

- **Anti-IC binding inhibition (1A7) for detection of anti-IC antibodies using 1A7-coated plates (Fig. 1C). This assay is identical to the anti-IC binding inhibition assay described in the paragraph above (for Fig. 1B) with the exception that the capture molecule is the murine 1A7 antibody. This antibody, specific for the idiotypic determinant of the 14.18 anti-GD2 antibody, binds to the 14.18 mAb, and thus serves as a mimic for GD2 (24).**
- **Anti-idiotypic binding inhibition for detection of anti-idiotypic antibody by using 1A7 mAb-coated plates and biotinylated hu14.18 mAb (Fig. 1D). The 1A7 mAb resembles the GD2 antigen and will capture or bind biotinylated hu14.18 antibody. The bound biotinylated 14.18 can then be quantified using the same biotin-avidin enzyme system used in the bridging assay described above (Fig. 1A). Briefly, C8 Maxisorp microtiter plates (Nunc) are coated overnight at 4°C with 120 μL of 2 μg/mL 1A7. Serum samples are diluted 1:5 with a solution of 3.1 ng/mL biotinylated hu14.18 and added to plates (100 μL/well). The plates are incubated overnight at 4°C and bound 14.18 is determined using the same biotin-ExtrAvidin-HRP enzyme system used above. Results are presented as % inhibition, where the amount of hu14.18 detected in pretreatment serum is defined as % inhibition for that patient, and % inhibition is calculated as for the assay presented in Fig. 1B.**
- **Anti–Fc-IL2 binding inhibition for detection of antibody to the Fc-IL2 region of the IC using anti–IL2–coated plates and biotinylated Fc-IL2 (Fig. 1E). Briefly, microtiter plates (Nunc) are coated overnight at 4°C with 120 μL of 2 μg/mL neutralizing rat monoclonal anti-human IL-2 antibody MQ1-17H12 (BD Pharmergen). Sample serum are diluted 1:5 with a solution of 5 ng/mL biotinylated hu14.18 Fc-IL2 fragment and added to plates at 100 μL per well. The plates are incubated overnight at 4°C and bound Fc-IL2 determined using enzyme system described above. Any decrease in the detected amount of Fc-IL2 from that expected reflects the presence of inhibitory activity (anti–Fc-IL2 antibody). Results are presented as % inhibition, where the amount of Fc-IL2 detected in pretreatment serum is defined as % inhibition for that patient, and % inhibition is calculated as for assays B and D above.**

**Statistical analysis.** The association between anti-idiotypic activity and IC dose used Pearson’s correlation analysis. The comparison of peak serum levels of hu14.18-IL2 between courses used Wilcoxon Signed Rank test. An exact McNemar’s test was used to compare anti-idiotypic and anti–Fc-IL2 response rates between courses. Logistic regression analysis was done to assess the dose response relationship between IC dose and anti–Fc-IL2 response. χ² analysis was used to evaluate the association between anti-IC antibodies and clinical toxicities. A two-sided significance level of 0.05 was used for all statistical tests.
Results

Detecting and quantifying anti-idiotypic responses. The anti-id-bridge assay, based on our previously developed human anti-chimeric antibody assay (23), was established to specifically see how well patients’ serum specimens (obtained at greater than or equal to thrice per 4-week treatment course) could bridge biotinylated hu14.18 mAb to hu14.18 bound to the plate (Fig. 1A).

Of 61 adult and pediatric patients evaluated in these phase I dose escalation studies, reflecting 126 courses of hu14.18-IL2 treatment, 28% were negative, 20% showed a minimal response, and 52% had a positive anti-idiotypic response with at least one specimen showing an OD reading of >0.7 No pre-treatment samples were positive and there was no correlation of anti-idiotypic activity and IC dose. The “anti-idiotypic” specificity of this assay was confirmed by showing that serum samples from patients that were able to “bridge” biotinylated hu14.18 mAb to hu14.18 bound to the plate could also bridge biotinylated hu14.18 mAb to a plate coated with ch14.18 mAb, but not to plates coated with Rituxan, Herceptin, KS1/4, R24, or UPC-10 humanized or murine mAbs (data not shown).

We next determined when the peak anti-idiotypic responses were detected. The adult study included 19 patients who received two courses of IC. Seven patients did not develop any “bridging antibody,” whereas 12 showed a detectable anti-idiotypic response. Five of these 12 showed their highest anti-idiotypic activity in course 1, 3 in course 2, and 4 had similar levels of anti-idiotypic bridging antibodies during course 1 and 2. In the pediatric study, there were 20 patients who received more than one treatment course. Three of these patients did not develop a bridging antibody (one patient with three courses and two patients with four courses). Seventeen developed detectable anti-idiotypic Ab, 9 had their highest OD value in their first course, 4 during their final course, and 4 during a course between their first and final courses. Thus, it seems that for this bridging anti-idiotypic assay, the highest values are more often observed after the first course than the last. Of 29 patients in the two studies that received more than one course and developed an anti-idiotypic Ab response, 14 had their highest OD value during course 1, whereas only 7 had their highest value after the last course. This indicates that, for most patients, continued exposure to IC does not continue to “boost” the anti-idiotypic response detected in this bridge assay.

The influence of anti-IC antibody on IC binding to GD-2. We tested whether the serum samples could inhibit hu14.18-IL2 IC from binding to GD2. We devised a GD2-based anti-IC ELISA (Fig. 1B; ref. 21) where serum from a patient is incubated with a fixed amount of IC to determine if anti-idiotypic antibody inhibits binding of hu14.18-IL2 to the plate-bound GD-2. This assay was used for the first 18 MEL patients accrued to the phase I study. Six patients showed measurable activity in the anti-IC-GD2 binding inhibition ELISA but no activity in the bridge assay (data not shown; Fig. 1A). This suggests that this binding inhibition assay (Fig. 1B) is more sensitive than the bridge assay, or that it may detect antibody not detectable in the bridge assay. It is possible that some high affinity and/or low level of anti-idiotypic antibody that can be detected in this GD2 binding inhibition assay may not be detected in the bridging ELISA, indicating a potential distinction in sensitivity for anti-idiotypic detection. The IC structure is such that two distinct types of anti-IC antibody might be detected in this GD2-based IC-binding inhibition assay. First, anti-idiotypic antibodies could prevent the IC from binding to the plate-bound GD2. Second, an antibody against the Fc-IL2 linkage region might interfere with detection of IC when using an anti-IL2 detection antibody (shown schematically in Fig. 1B). To address this, we developed two additional ELISA assays that independently detected anti-idiotypic antibody or antibody against the Fc-IL2 linkage component. These are the anti-idiotypic binding inhibition (Fig. 1D) and anti–Fc-IL2 binding inhibition (Fig. 1E) assays.

Anti-idiotypic antibodies. The anti-idiotypic binding inhibition assay was designed to detect an antibody specific for the idiotypic determinant. Patients’ serum was added to biotinylated hu14.18 mAb, before the ELISA capture step (Fig. 1D). The capture antibody is the murine IAT mAb (24), specific for the 14.18 antibody idiotypic determinant. When patients have

<table>
<thead>
<tr>
<th>Anti id-Bridge</th>
<th>Anti IC-Binding Inhibition (GD-2)</th>
<th>Anti IC-Binding Inhibition (1A7)</th>
<th>Anti id-Binding Inhibition</th>
<th>Anti Fc IL2 Binding Inhibition</th>
</tr>
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<tr>
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<td><img src="image2.png" alt="Diagram" /></td>
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<td><img src="image4.png" alt="Diagram" /></td>
<td><img src="image5.png" alt="Diagram" /></td>
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Fig. 1. ELISA methods to detect antibodies to the hu14.18-IL2 IC. A, anti-Id-Bridge detects anti-idiotypic antibody in patient serum bridging biotinylated hu14.18 antibody to plate-bound 14.18 antibody. B, anti-IC binding inhibition (GD-2) detects anti-idiotypic antibody in patient serum interfering with hu14.18-IL2 IC binding to GD2-coated ELISA plates and also detects anti-Fc-IL2 antibody in patient serum that interferes with anti-IL2 detection of IC bound to GD2-coated plates. C, anti-IC binding inhibition (1A7) detects both anti-idiotypic and anti–Fc–IL2 antibodies, as in B, with the exception that the capture reagent is plate-bound murine anti-idiotypic 1A7, which specifically recognizes the 14.18 idiotypic determinant. D, anti-id binding inhibition detects anti-idiotypic antibody in patient serum interfering with binding of biotinylated hu14.18 to plate-bound 1A7. E, anti–Fc–IL2 Binding inhibition detects antibody in the patient serum to a determinant on the IC where the IL-2 is genetically linked to the carboxyl terminus of the IgG1 heavy chain (anti-Fc-IL2 antibody). This antibody in patient serum blocks the binding of the biotinylated Fc-IL2 fragment to plate bound anti-IL2 antibody.
Although the magnitude of the anti-idiotypic response detected in this binding inhibition assay was not influenced by the dose of hu14.18 administered, it was influenced by the number of courses the individual patients received. This was noted in the pediatric study where 28 patients received from one to four courses of hu14.18-IL-2. The percentage of patients developing >50% inhibition in the anti-idiotypic binding inhibition assay increased with the number of courses of IC administered (Table 1). This change in reactivity was significant between course 1 and subsequent courses (course 1 versus course 2, 3, or 4, \( P = 0.002 \), \( P = 0.026 \), \( P = 0.007 \), respectively), as well as when comparing course 1 to courses 2, 3, and 4 combined (\( P \leq 0.001 \)). This contrasts with the results of the bridging anti-idiotypic ELISA, where more patients developed their highest bridging activity during their first course.

We next asked whether the induction of anti-idiotypic antibody as detected by this anti-idiotypic binding inhibition ELISA (Fig. 1D) would also influence the serum levels of hu14.18-IL-2 measured during a second course of treatment. When data from 11 evaluable adult melanoma patients, who received two courses of IC at the same dose for each course were analyzed, those patients that showed greater inhibitory activity in this binding inhibition assay also showed a corresponding decrease in their peak IC serum level measured immediately after finishing the 4-hour infusion on C2D1 compared with their peak level obtained immediately after the 4-hour infusion of the same IC dose on C1D1 (\( R = 0.83; P = 0.002 \); data not shown). This indicates that the patients with the strongest anti-idiotypic antibody responses show the lowest detectable serum IC levels during the first day of their second course of treatment.

**Anti–Fc-IL-2 antibodies.** A separate “anti–Fc-IL-2 binding inhibition” assay was designed to detect antibody specific for the Fc-IL-2 linkage component of the IC (Fig. 1E). Serum specimens were added to biotinylated Fc-IL-2 fragments before their capture on the ELISA plate coated with an anti-IL-2 antibody, followed by detection with ExtrAvidin-HRP. If the patient made an antibody that bound to this fragment, it might interfere with the binding of the Fc-IL-2 to the capture anti-IL-2 antibody. Data from all 33 adult MEL patients are shown for course 1 in Fig. 2B. C1D8 serum from 32 of 33 patients showed development of detectable inhibitory activity. Table 2 shows representative anti-IC values in the three binding inhibition assays (Fig. 1B-D) for two patients with strong anti-idiotypic responses. Separate assays (data not shown) evaluate the functional effects, and the specificity, of the anti IC antibodies detected in these ELISA assays, and will be presented in a subsequent report.\(^4\) There was no relationship between the magnitude of the anti–Fc-IL-2 response and the level of hu14.18-IL-2 detected in the serum during course 2 (\( R = 0.272; P = 0.41 \)). A dose-response relationship was identified for this anti–Fc-IL-2 response; patients receiving higher doses of IC show stronger anti–Fc-IL-2 responses measured on day 8 and day 15 in course 1 (\( P = 0.005 \); data not shown). Similarly there was a significant positive correlation between the area under the curve for serum concentration of hu14.18-IL-2 on C1D1 and the development of anti–Fc-IL-2 antibodies. The biological mechanism for these results requires further evaluation, but might suggest that greater doses of hu14.18-IL-2 or greater

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\(^4\) Hank, et al., in preparation.
exposures to hu14.18-IL2 were more potent at inducing the anti-Fc-IL2 response.

Clinical correlations with anti-IC antibody. Data from two representative NBL patients receiving four courses of hu14.18-IL2, one with and one without an antibody response to the IC, are presented in Fig. 3. Patient 10 generated very little anti-idiotypic antibody (Fig. 3A) and the amount of IC detected in the serum was similar for all four courses (serum IC levels were measured on days 1 and 3 of each treatment course). In contrast, patient 19 developed anti-IC antibodies during the first course of treatment, which were evident in the binding inhibition assay by day 8 of course 1 (Fig. 3B). Anti-idiotypic antibodies were also noted in the bridging assay following courses 2, 3, and 4. The serum IC levels detected during courses 2, 3, and 4 were markedly lower than the levels seen in course 1, indicating that the anti-IC response was associated with lower detectable IC. The lymphocyte count and serum level of sIL2Rα are both indicators of immune activation induced by IL2 (25). Although the level of IC detected in serum from patient 19 was lower in courses 3 and 4 than in course 1 (presumably due to the anti-IC response), the lymphocytosis and increase in sIL2Rα in courses 3 and 4 were comparable with that seen in course 1 (Fig. 3D). Thus, administration of IC in courses 3 and 4 to patient 19 still resulted in comparable IL2-induced in vivo activation (lymphocytosis and increase in sIL2Rα) despite the relative inability to detect circulating IC in the ELISA. Patient 10 generated very little anti-idiotypic antibody and values for lymphocyte counts, and sIL2Rα increased and remained elevated throughout the four courses of therapy (Fig. 3C).

As noted above, greater anti-idiotypic responses were associated with an inhibition in the detected levels of IC on C2D1 (P < 0.002). Either the circulating anti-idiotypic Ab in these patients is removing the IC from the serum or preventing it from being detected in the ELISA. Data from patient 19 in the pediatric trial (Fig. 3) shows that the anti-idiotypic response may be preventing IC detection, as well as removing some IC from the circulation. The serum levels of hu14.18-IL2 immediately after the 4-hour IC infusion (Fig. 3) have been measured on days 1 and 3 for each of the four courses.

The levels of anti-idiotypic and anti-FcIL2 antibody were evaluated in the MEL study for any relationship with the previously published hu14.18-IL2 induced changes: (a) change in lymphocyte counts, (b) change in lymphocyte phenotype (increase in % CD16+ and CD56+ and decrease in % CD3+ peripheral blood mononuclear cells), and (c) increase in serum sIL2R level (14). No significant correlations were seen between the anti-idiotypic response and these three parameters. The anti-Fc-IL2 response did not correlate with lymphocyte numbers or phenotype, but patients that induced the strongest anti-Fc-IL2 antibody were also those that showed a greater increase in their sIL2Rα levels in course 2 than in course 1 (P < 0.01). The mechanism for this relationship remains uncertain.

Discussion

Treatment of adult and pediatric patients with IC induces antibody responses to IC in some but not all patients. Some patients initially make a low level of antibody that does not increase progressively with increasing number of courses. Others make antibody responses that are more detectable with subsequent courses of treatment. This phenomenon was previously reported by Welt et al. (26) who treated 11 patients with advanced colorectal cancer with the humanized IgG1 A33 antibody. Of these 11 patients, 3 did not develop an antibody to A33 antibody. The eight patients developing a “human anti-humanized antibody” (HAHA) response, were divided into two groups. Five patients developed a type I HAHA response, which generally developed early, within 2 weeks of treatment.

Table 1. Time course for development of anti-Id and anti-Fc-IL2 antibodies in pediatric patients

<table>
<thead>
<tr>
<th>Course</th>
<th>Anti-ID inhibition</th>
<th>Anti-Fc-IL2 inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(+++)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
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<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

NOTE: The % of pediatric patients with anti-idiotypic and anti-FcIL2 antibodies seen in the binding inhibition ELISAs following each of four courses of IC. ++, >50% inhibition in the anti-idiotypic and >40% inhibition in the anti-FcIL2 ELISA, respectively.

Table 2. Anti-hu14.18-IL2 activity (% inhibition) detected in binding inhibition assays

<table>
<thead>
<tr>
<th>Day of serum collection</th>
<th>Patient 5</th>
<th>Patient 9</th>
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<tbody>
<tr>
<td></td>
<td>C1D8</td>
<td>C1D15</td>
</tr>
<tr>
<td>Anti-idiotypic binding inhibition</td>
<td>93</td>
<td>90</td>
</tr>
<tr>
<td>Anti-Fc-IL2 binding inhibition</td>
<td>43</td>
<td>34</td>
</tr>
<tr>
<td>Anti-IC binding inhibition (GD2)</td>
<td>87</td>
<td>95</td>
</tr>
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</table>

NOTE: The % inhibition in the ability to detect a standard amount of biotinylated hu14.18 (anti-idiotypic, as shown in Fig. 1D), Fc-IL2 fragment (anti-Fc-IL2, as shown in Fig. 1E), or IC (anti-IC, as shown in Fig. 1C) in ELISA when combined with serum samples obtained on day 8 or 15 of the first or second course of therapy with hu14.18-IL2 IC. All % inhibition values are based on the pretreatment serum sample for each patient (defined as 0% inhibition for that patient). Values shown are % inhibition for two patients, treated with two courses of hu14.18-IL2 in the adult MEL study that showed greater than average inhibitory activities in these three assays.
and usually resolved within 7 weeks of treatment and did not progress with the number of courses. Three patients developed a type II HAHA response, characterized by progressively increasing titers of anti-huA33 antibodies. We noted similar trends in anti-IC responses. Some patients developed antibodies that increased in magnitude with the number of courses (i.e., patient 19; Fig. 3). Some patients developed detectable levels of anti-IC antibody, but the strength of the response did not continue to increase, or did not seem to affect the level of IC detected in the patient serum following subsequent IC infusions (i.e., patient 10; Fig. 3). Although it seems that patient 19 developed a response that is similar to the type II response described by Welt et al. (26), the significance of this response in these IC-treated patients is not yet known, as the generation of an antibody was not associated with increased toxicity upon subsequent infusions of IC.

In Fig. 3 (and in data not shown), anti-IC antibodies that are detected in the binding inhibition ELISA limit the detection of IC in patient serum. Of interest are the anti-idiotypic antibodies measured by the bridging assay on days immediately following each 3-day course of IC. In Fig. 3B, patient 19 shows high OD values corresponding to the levels of anti-idiotypic bridging antibody at C2D8 and all subsequent time points, except for the striking drop in activity seen on day 4 of courses 3 and 4. These serum samples are obtained 20 hours following the completion of the third IC infusion. As the half-life of the IC is 3.1 to 3.7 hours (14, 17), circulating IC should not be influencing the results of the course 3 and 4 day 4 bridging assay. The major drop in the anti-idiotypic antibody seen in courses 3 and 4 on day 4 compared with the day 1 value suggests that the 10 mg/M2 of hu14.18-IL2 infused daily on days 1, 2, and 3 of that course may have “complexed” with the majority of the circulating anti-idiotypic antibody, thereby limiting the amount of anti-idiotypic antibody that could be detected on day 4. The peak IC serum levels during courses 1 and 2 are ~4 μg/mL, whereas during courses 3 and 4, serum IC was virtually undetectable. This absence of detectable IC following an IV hu14.18-IL2 infusion suggests that the anti-IC response was able to “neutralize” virtually all of the hu14.18-IL2 given to this patient at that time. Similarly, the anti-idiotypic response measured with the bridge ELISA on day 4 of courses 3 and 4 is only ~20% of that seen on day 1 of these same two courses, suggesting that infusing hu14.18-IL2 that generated a peak serum level of 4 μg/mL during course 1 and 2 was able to “neutralize” most of the circulating anti-IC antibody. This suggests that the serum level of functional anti-IC antibody might also be near 4 μg/mL. Because our other methods of anti-idiotypic detection require binding inhibition assays, where the inhibiting anti-IC antibody is in excess, it is impossible to determine the actual concentration (i.e., μg/mL) of anti-IC antibody. The antibody to the Fc-IL2 determinant does not completely inhibit Fc-IL2 binding to anti-IL2 mAb in the ELISA. Unlike the anti-idiotypic assays, where % inhibition values were occasionally approaching 100%, % inhibition values in the anti-Fc-IL2 assay rarely exceeded 90% (see Table 2). Furthermore anti-Fc-IL2 inhibitory activity detected in these ELISA is readily lost with a 10-fold dilution of the serum sample (data not shown). In contrast, anti-idiotypic Ab can completely inhibit specific binding of the hu14.18 antibody to GD2-coated (Fig. 1B) and 1A7-coated (Fig. 1C) ELISA plates. In some
patients, serum samples continue to inhibit at dilutions as great as 1:100. However, in two pediatric patients with considerable inhibition in the anti-idiotypic binding inhibition assay throughout four courses of treatment, the serum sample with the most potent inhibition (and the ability to retain inhibitory activity with 1:100 dilution) was noted in the second, rather than third or fourth course (data not shown), suggesting some waning of the anti-idiotypic response.

The clinical significance of the anti-idiotypic antibodies is yet to be determined. Of these 61 melanoma and neuroblastoma patients, 52 developed antibodies detected in either the anti-idiotypic bridge or anti-idiotypic binding inhibition assays. Of these 52, 32 patients were positive in both assays, 8 were positive only in the binding inhibition assay, and 12 were positive only in the bridge assay. These anti-idiotypic antibodies were not associated with an increase in toxicity or allergic phenomena (14, 17). Nor were these anti-idiotypic antibodies associated with changes in clinical laboratory markers or markers associated with IL-2 induced immune activation (sIL-2Rα and increase in the lymphocyte count). It is not clear if the amount of anti-IC antibody present in the serum would be sufficient to limit the ability of the IC to target GD2-positive tumors in vivo and, if so, what levels of reactivity in vitro would correspond to meaningful in vivo inhibition of function. Our preliminary data indicate that levels of anti-IC antibody detected for most patients in the ELISA reported here do not necessarily correspond to levels that inhibit IC binding to GD2 or IL2R in conditions designed to simulate the in vivo setting. The development of anti–Fc–IL2 antibody also was not associated with an increase in toxicity or change in the lymphocyte count, but there was a significant hu14.18-IL2 dose effect and correlation of anti–Fc–IL2 response with the AUC for hu14.18-IL2. This suggests that greater exposure to hu14.18-IL2 induces more anti–Fc–IL2 antibodies. In addition, patients that induced the strongest anti–Fc–IL2 antibody were those that showed a greater increase in their sIL2R levels in course 2 than in course 1. This indicates an association between stronger activation of sIL2R in a patient’s second course with induction of a strong anti–Fc–IL2 antibody. The mechanism of this requires further study, as do its clinical implications.

Although anti-idiotypic antibodies can interfere with the desired antigen binding function of the mAb, at least in vitro, it remains controversial whether an anti-idiotypic response might be beneficial or harmful for the therapeutic effect desired in treated patients. In some clinical studies of mAb used as cancer therapy, the presence of an anti-idiotypic antibody response, particularly if transient, has been associated with an improved antitumor effect (27–29). This improved effect has been postulated to result from the induction of an “anti–anti-idiotypic” response, designated an “antibody-3 response,” which may have direct antitumor activity. In fact, clinical efforts to induce a potent antitumor response have used immunization with anti-idiotypic antibodies as vaccines, designed to activate a tumor reactive antibody-3 response (24, 29). Our initial efforts to detect antibody 3 responses in selected patients showing strong anti-idiotypic responses have not revealed antibody 3 activity (data not shown). It remains uncertain whether the induction of the anti-idiotypic and anti–Fc–IL2 antibodies might help or interfere with the desired in vivo clinical effect. As there were no clinical responses noted in these phase I studies, it is impossible to predict the impact of the generation of antibody response to the IC upon clinical outcome. We have just completed phase II trials of this agent for 14 adults with MEL and 39 children with NBL; antitumor activity was seen in each study (30, 31). We are now performing the in vitro analyses of anti-IC responses for these 53 patients.

In summary, anti-IC antibodies were formed in most patients. These included HAHA responses to the idiotypic determinant and antibodies specific for a neoantigen created at the Fc–IL2 junction site of the IC molecule. These antibodies do not seem to induce allergic reactions or increase the toxicity of the IC. The in vivo functional significance of this antibody response is yet to be determined. The lymphocytosis and sIL2R levels seen in the face of highly reactive anti–Fc–IL2 antibody, suggest that these anti-IC antibodies do not inhibit IL-2–induced immune activation in vivo. Additional studies are under way to better characterize the in vivo effects of these anti-idiotypic and anti–Fc–IL2 antibodies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

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