Humoral Immune Function in Pediatric Patients Treated With Autologous Bone Marrow Transplantation for B Cell Non-Hodgkin's Lymphoma. The Influence of ex vivo Marrow Decontamination With Anti-Y 29/55 Monoclonal Antibody and Complement

By Christoph Baumgartner, Andreas Morell, Andreas Hirt, Urs Bucher, Hansjörg K. Forster, Jan E. Doran, Lukas Matter, Guido Brun del Re, and Hans P. Wagner

Elimination of neoplastic B cell populations from autologous bone marrow grafts also removes normal B lymphocytes. This is potentially hazardous for the reconstitution of the immune system in patients undergoing high-dose chemotherapy and total body irradiation followed by autologous marrow rescue. Five pediatric patients with B cell non-Hodgkin's lymphoma in first remission undergoing such a regimen were studied. They received bone marrow pretreated with anti-Y 29/55 monoclonal antibody and complement. B and T lymphocyte subpopulations reached normal levels within 6 months after autologous bone marrow transplantation (ABMT), and serum immunoglobulin levels became normal within 4 to 9 months. Vaccination with diphtheria and tetanus toxoid, trivalent poliomyelitis vaccine of the Salk type, and pneumococcal capsular antigens (38 to 54 months after transplantation) gave rise to specific antibody production. ABO isoagglutinins could be demonstrated in all patients. The response pattern was similar to that of patients who received unmanipulated autologous bone marrow. It is concluded that ex vivo anti-Y 29/55 depletion of the marrow graft does not induce relevant disturbances of humoral immune functions.

All patients had received basic immunization against diphtheria, tetanus, and poliomyelitis (but not against pneumococcal antigens) before diagnosis of B cell non-Hodgkin's lymphoma. Some patients had been revaccinated for diphtheria, tetanus, and poliomyelitis in the interval between ABMT and the present immunization studies (Tables I and 2). All patients were negative for anti-HIV at the time of these studies and had not received immunoglobulin containing preparations for at least two years. The present study was performed with official institutional approval. Patients were included only after informed consent was given by themselves and/or their parents.

Chemoradiotherapy and Supportive Care

After initial surgery, remission was induced with a regimen as previously described including vincristine, adriamycin, cyclophosphamide, prednisone, and triple intrathecal chemotherapy. Cranial irradiation (20 to 24 Gy) was given to all patients with CNS involvement. Ten to 14 weeks after diagnosis remission was consolidated by a regimen with vincristine 2 mg/m², adriamycin 60 mg/m², cyclophosphamide 45 mg/kg four times, and total body irradiation (TBI, single dose) of 6 Gy in midplane, followed by ABMT. TBI was delayed with anti-V 29/55 monoclonal antibody and rabbit complement.5 Five of six patients transplanted with decontaminated marrow and four of eight patients with untreated marrow became long-term survivors. Elimination of B cells from the graft raises the concern of impaired B cell reconstitution and function after ABMT. This study provides data on the humoral immune function of these patients and compares it with that of similar patients receiving untreated marrow.

PATIENTS, MATERIALS, AND METHODS

Patients

Five surviving male patients diagnosed to have non-Hodgkin's lymphoma of small noncleaved cell, Burkitt's type (NCI working formulation), transplanted in first remission with anti-Y 29/55 and rabbit complement.5,6 Five of six patients transplanted with decontaminated marrow and four of eight patients with untreated marrow became long-term survivors. All patients had received basic immunization against diphtheria, tetanus, and poliomyelitis (but not against pneumococcal antigens) before diagnosis of B cell non-Hodgkin's lymphoma. Some patients had been revaccinated for diphtheria, tetanus, and poliomyelitis in the interval between ABMT and the present immunization studies (Tables I and 2). All patients were negative for anti-HIV at the time of these studies and had not received immunoglobulin containing preparations for at least two years. The present study was performed with official institutional approval. Patients were included only after informed consent was given by themselves and/or their parents.

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delivered by a cobalt source. The irradiation time was kept as short as possible by adjusting the source skin distance, resulting in a dose rate between 2.5 and 10 cGy/min in midplane. We did not correct for inhomogeneities of ± 15% as measured by TLD (TLD-100; Harshaw, Solon, OH). Patients were kept in reverse isolation and received cotrimoxazole and non-absorbable anti-mycotics orally and cell support as described earlier. All patients were given a subsituation of intravenous (IV) immunoglobulin (Sandoglobulin, Sandoz, Basel, Switzerland) 0.4 g/kg every 2 weeks until normalization of blood cell counts, then every 4 weeks until 3 to 4 months after ABMT.

In Vitro Bone Marrow Treatment

Anti-Y 29/55 monoclonal antibody. The generation of the mouse hybridoma secreting the monoclonal antibody anti-Y 29/55 has been described previously. The antibody belongs to the class IgG 2A kappa and binds rabbit complement. It recognizes a membrane bound glycoprotein with a putative molecular weight of 70 kd. This antigen is confined to human B lymphocytes. Its appearance on the cell surface is related to maturation. Pre-B cells are negative, approximately 50% of sIgM+ sIgD- cells and approximately 98% of sIgM+, sIgD- cells bind anti-Y 29/55. Hematopoietic cells from human bone marrow including granulocyte-macrophage precursors (CFU-GM) do not react with this antibody. Regardless of surface immunoglobulin expression anti-Y 29/55 used in indirect immunofluorescence stained practically all tumor cells of all 12 pediatric B cell non-Hodgkin's lymphomas examined. Antibody containing ascites fluid was produced in BALB/c J mice after intraperitoneal inoculation with 3 x 10^6 anti-Y 29/55 hybridoma cells. Ascites fluid was collected and processed by several centrifugation steps including ultracentrifugation. It was then passed through 0.22 μm sterile filters and transferred into sealed vials under galenic conditions. A titer of 1/6000 was determined in a modified microcytotoxicity assay with Daudi cells as targets. The material proved to be sterile and free of pyrogens, reverse transcriptase and RNA-dependent DNA polymerase activity. It was stored at -160°C until use.

Complement. Unpooled rabbit serum from 2- to 4-month-old animals was used as the complement source. The preparation included ultracentrifugation, sterile filtration (0.45 μm pore size) and storage in ampoules at -160°C. This material was assessed for complement activity, sterility, and absence of pyrogens and toxicity for normal human bone marrow cells (dye exclusion test and CFU-GM cultures). Bone marrow processing and incubation conditions. Bone marrow was harvested after one or two courses of chemotherapy. At this time no tumor cells were detected in multiple routine smears and biopsies of the marrow. Bone marrow was aspirated under general anesthesia from the posterior and anterior iliac crest and occasionally from the sternum. The aspirate was anticoagulated with preservative-free heparin. After passage through a standard blood transfusion set the buffy coat was obtained. Mononuclear cells were then separated by discontinuous density gradient centrifugation (ficolmitroza, Lymphoprep [Nygard and Co, Oslo]; density 1.077). This cell fraction was suspended in human plasma protein solution (5 g/dL). Antibody (3 to 6 mL ascites fluid per 100 mL cell suspension) and complement (20 to 25 mL/100 mL final concentration) were added resulting in a final concentration of 15 to 30 x 10^6 cells/mL. This cell suspension was incubated for 90 minutes at room tempera-

Table 1. Antibody Response to Tetanus and Diphtheria Vaccination in Patients With ABMT

<table>
<thead>
<tr>
<th>Patients</th>
<th>Tetanus Antitoxin</th>
<th>Diphtheria Antitoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutralization (IU/mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Before Vaccination After Vaccination</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.0* &gt;5.0</td>
<td>3.5* &gt;8.5</td>
</tr>
<tr>
<td>3</td>
<td>0.1 0.5</td>
<td>0.45 0.45</td>
</tr>
<tr>
<td>4</td>
<td>3.0* &gt;5.0</td>
<td>0.45* &gt;8.5</td>
</tr>
<tr>
<td>5</td>
<td>0.1 0.1</td>
<td>0.46* 2.75</td>
</tr>
<tr>
<td>A</td>
<td>0.02* &gt;10</td>
<td>2.8* 6.2</td>
</tr>
<tr>
<td>B</td>
<td>&lt;0.1* &gt;5.0</td>
<td>&lt;0.42* &gt;8.5</td>
</tr>
<tr>
<td>C</td>
<td>0.1 0.5</td>
<td>0.45 0.45</td>
</tr>
</tbody>
</table>

Antitoxin levels above 0.01 IU/mL are considered to be protective.
*Patient no. 1 was not available for immunization studies.
†Patient no. 1 was not available for immunization studies.

Table 2. Neutralizing Antibody Response to Poliomyelitis Vaccination (Trivalent Salk Vaccine) in Patients Treated With ABMT

<table>
<thead>
<tr>
<th>Patients</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Vaccination</td>
<td>3-4 Weeks After Vaccination</td>
<td>Before Vaccination</td>
</tr>
<tr>
<td>2*</td>
<td>64</td>
<td>512</td>
<td>32</td>
</tr>
<tr>
<td>3*</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>4*</td>
<td>32</td>
<td>256</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>A*</td>
<td>256</td>
<td>9192</td>
<td>128</td>
</tr>
<tr>
<td>B*</td>
<td>8</td>
<td>32</td>
<td>18</td>
</tr>
<tr>
<td>C*</td>
<td>16</td>
<td>1024</td>
<td>32</td>
</tr>
</tbody>
</table>

Tests were done in triplicate and median values are listed here. Reciprocal titers > 16 are considered to be protective.
*Patients revaccinated after ABMT and before the present immunization studies.
†Patient no. 1 was not available for immunization studies.
The darkness of the lines was measured photometrically, reflecting in which standardized quantities of phase enzyme immunoassay fled by peroxidase-labeled rabbit anti-human IgG, IgM, and IgA, applied to nitrocellulose. Specifically bound antibodies were identified pneumococcal antigens (type 1, 2, 3, 4, 6, 8, 9, and 12) were positive RBCs. Test with serial dilution and macroscopic ABO isoagglutinins were determined in a manual hemagglutination test with reading using a microtiter system with African Green type mouse neutralization test with final reading of the mice four days after inoculation. Tetanus antitoxin in the rabbit skin test with reading of the results 48 hours after vaccination before and 3 to 4 weeks after vaccination. Co. mc, Rahway, NJ). Antibody levels or titers in plasma were used as comparison with a laboratory and the TCID doses were calculated by a standard preparation.16

and in an enzyme linked immunosorbent assay after inoculation.14

Determination of lymphocyte subpopulations. Routine hematological methods were used daily to assess recovery of peripheral blood cell counts. Subpopulations of blood lymphocytes were studied before the ABMT procedure, at the end of hospitalization, and then at intervals as allowed by the clinical follow-up examinations. Mononuclear cells were separated by ficoll-hypaque centrifugation and analyzed by indirect immune fluorescence microscopy. Absolute blood levels were calculated from the percentage of reacting lymphoid cells and the differential WBC count. The following reagents were used: OKT3, OKT4, OKT8 (Ortho Diagnostics, Raritan, NJ), Anti-Leu-1, Anti-Leu-2a, Anti-Leu-3a (Becton Dickinson, Mountain View, CA), FITC-labeled anti-human Ig and FITC-labeled goat anti-mouse Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands).

Evaluation of humoral immune function. Serum immunoglobulin levels (IgG, IgM, and IgA) were determined quantitatively using standard laboratory methods of radial immunodiffusion. The patients were immunized with diphtheria and tetanus toxoid, trivalent salt-type poliomyelitis vaccine (Di Te Anatoxal Berna and Poliomyelitis-Impfstoff Berna, Swiss Serum and Vaccine Institute, Bern, Switzerland), and pneumococcal capsular antigens (Moniarix, Smith Kline and French, Philadelphia or Pneumovax, Merck & Co, Inc, Rahway, NJ). Antibody levels or titers in plasma were measured before and 3 to 4 weeks after vaccination.

Diphtheria antitoxin was measured by the intracutaneous method in the rabbit skin test with reading of the results 48 hours after inoculation.15 Tetanus antitoxin was determined in the standard mouse neutralization test with final reading of the mice four days after inoculation and in an enzyme linked immunosorbent assay (ELISA).15

Antibodies to polioviruses were determined in a tissue culture neutralization test using a microtiter system with African Green monkey kidney cell cultures as indicator cells. The following viruses were used as challenge virus: type 1, Charleston; type 2, MEF-1; type 3, Saukett. Readings were made eight days after inoculation and the TCID₉₀ doses were calculated by comparison with a laboratory standard preparation.16

Antibodies to pneumococcal antigens were determined by a solid phase enzyme immunoassay in which standardized quantities of eight pneumococcal antigens (type 1, 2, 3, 4, 6, 8, 9, and 12) were applied to nitrocellulose. Specifically bound antibodies were identified by peroxidase-labeled rabbit anti-human IgG, IgM, and IgA. The darkness of the lines was measured photometrically, reflecting the amount of class specific serum antibody bound to the antigen.16,18

ABO isoagglutinins were determined in a manual hemagglutination test with serial dilution and macroscopic reading using A, and B positive RBCs.

**Statistics.** Data of the humoral response to pneumococcal capsular antigens were analyzed by the use of analysis of variance techniques. Since antibody reactivity of the same individual was measured more than once, a repeated measures design was used. The response of each antibody class (IgG, IgM, and IgA) was considered separately.

**RESULTS**

**Lymphocyte and Immunoglobulin Recovery**

Recovery of blood lymphocytes and their subpopulations after transplantation of anti-Y 29/55 depleted marrow is shown in Fig 1. Normal levels of B cells (>0.1 x 10⁹/L) were reached 6 months after ABMT. Recovery of T lymphocytes was more variable. Decreased levels (<0.57 x 10⁹/L) were observed up to 9 months after ABMT. Inversion of the T4/T8 ratio (<1.0) occurred in all five patients and persisted in three for more than 18 months and in one for more than 52 months.

Recovery of serum immunoglobulin levels is shown in Fig 2. IgG levels were maintained within normal range without substitution 4 months after ABMT. A period of 6 months was required for normalization of IgM, while decreased levels of IgA were observed up to about 9 months after ABMT.

**Production of Specific Antibodies**

Immunization studies were performed 38 or more months after ABMT in four patients who were reconstituted. The data are shown in Table I. The proportion of each antibody class (IgG, IgM, and IgA) was considered separately.

![Figure 1](image1.png) **Blood counts of total lymphocytes and subpopulations and T helper/T suppressor ratio after ABMT with anti-Y 29/55 and complement-treated marrow in five pediatric patients with B cell non-Hodgkin's lymphoma. The lower limit of the normal range is 0.8 x 10⁹/L for total lymphocytes, 0.1 x 10⁹/L for B cells, and 0.57 x 10⁹/L for T cells.**
virus were already in a protective range (>16) in six of 12 determinations in patients with anti-Y 29/55 treated marrow and in four of nine determinations in patients with untreated marrow (Table 2). A relevant increase of the titer against at least one type of poliovirus was observed in three of four patients with anti-Y 29/55 depleted marrow and in all three control patients (Table 2).

Antibody levels against eight different types of pneumococcal capsular antigens were assessed separately for the three antibody classes IgG, IgM, and IgA. Data for IgG are shown in Fig 3. IgG, IgM, and IgA antibody levels after immunization were significantly higher than baseline levels in both patient groups (F ratios for constant terms for IgG, IgM, and IgA are 62.92, 121.24, and 114.68, respectively, with 1,35 DOF: all P < .001). No significant differences were found in the responses of the two treatment groups.

Three patients failed to respond to one of the three antigen groups. Two patients, one of each treatment group, showed no or only a minimal response to bacterial toxoids (patients no. 3 and C, Table 1) while their reaction to polioviruses and pneumococcal capsular antigens remained unaffected. One patient with B cell depleted marrow did not respond to polio vaccination (patient no. 5, Table 2) while he was able to produce antibodies against the two other antigen groups. In contrast to all responsive patients, these patients had not been reexposed to the particular antigens since ABMT (Tables 1 and 2).

In addition, titers of ABO isoagglutinins were determined. In patients receiving anti-Y 29/55 depleted marrow anti-B titers were between 1/4 and 1/16 and anti-A, was 1/256. In the control group anti-B was 1/4 to 1/16 and anti-A, was 1/256 (normal range for anti-B, 1/4 to 1/16; for anti-A, 1/4 to 1/12).

DISCUSSION

In vitro manipulations of bone marrow involving specific antibodies are currently used to remove residual malignant cells before autologous transplantation in different neoplastic conditions. The monoclonal antibody anti-Y 29/55 used in this study induces complement mediated lysis of B lymphoma cells and of mature B cells at various stages of differentiation. Normal recovery of WBC, granulocytes,
thrombocytes, and reticulocytes after transplantation of anti-Y 29/55 depleted marrow has been demonstrated earlier.3 The present investigations addressed the question whether removal of immunocompetent B cells from the marrow graft impairs the humoral immune function after ABMT.

Blood B lymphocytes were already markedly depressed before ABMT. They were shown to reappear in all patients reconstituted with anti-Y 29/55 depleted marrow. Normal levels were reached in all cases within 6 months after ABMT. This is similar to what has been observed after transplantation of unmanipulated syngeneic or allogeneic marrow.22,23 The pattern of T cell recovery also resembled the one expected after transplantation of unmanipulated bone marrow.23-25 It included predominance of suppressor T cells with a low T4/T8 ratio (<1.0) which persisted for 4 to >52 months.

Serum immunoglobulin levels were also depressed before ABMT was started. After anti-Y 29/55 depleted ABMT IgG and IgM levels returned to normal within 4 to 6 months, while IgA levels were low for a period of approximately 9 months. Similar or even longer depressions of immunoglobulin levels have been reported for recipients of syngeneic or allogeneic marrow.22,23 The recovery of serum immunoglobulin levels was observed by other investigators in patients transplanted with autologous marrow pretreated with the anti-B cell antibody anti-B1, and complement.21 In a recent study the same group reports on 24 patients receiving an anti-B1 depleted marrow autograft.28 Though the relative number of blood B cells normalized within 5 to 6 months in these patients some of them had depressed serum IgG, IgA, and IgM levels and reduced blood T cell numbers for more than 1 year. The more sustained suppression of immune functions in this series as compared with ours may be related to the different cell spectra recognized by the two antibodies used for in vitro marrow purging. While anti-B1 also binds to pre-B cells anti-Y 29/55 is restricted to mature surface immunoglobulin bearing B cells and does not affect their precursors.5

Immunization studies were performed with three categories of antigens, ie, bacterial toxoids (diphtheria and tetanus toxoid), viral antigens (trivalent polio vaccine of the Salk type), and bacterial polysaccharide antigens (pneumococcal capsular antigens). The studies were done in parallel in a group of patients grafted with in vitro anti-Y 29/55 depleted marrow (38 to 54 months earlier) and a control group grafted with untreated marrow (72 to 87 months earlier).

The humoral response to diphtheria and tetanus toxoid was similar in both groups. In all cases pre-study levels were already in a protective range (>0.01 IU/mL). The majority of patients was able to increase the levels of specific antibodies to >2 IU/mL while one patient in each group showed little or no response to both antigens. Unlike all others the unresponsive patients had not been revaccinated between ABMT and the present immunization studies.

Specific antibody formation after trivalent polio vaccination was observed in both groups of patients. The response tended to be stronger in patients who had received untreated marrow. We assume that this is the result of the longer interval between ABMT and the present studies in this patient group (72 to 87 months as compared with 38 to 54 months for patients with anti-Y 29/55 depleted marrow). One patient who had received anti-Y 29/55 depleted marrow did not
respond to any of the three poliovirus types. His unresponsiveness may be explained by the fact that his interval between ABMT and the evaluation of humoral immune function was the shortest (38 months) of all patients in this study and that he was the only one who had not been revaccinated for poliomyelitis between ABMT and the present immunization studies.

Immunization with pneumococcal capsular antigens induced specific IgG, IgM, and IgA responses in all patients irrespective of in vitro bone marrow manipulation. The capacity to produce ABO blood group isoagglutinins was maintained in all patients and was independent of marrow manipulation.

These data show that the ABMT patients of this study are able to give rise to specific humoral immune responses irrespective of ex vivo marrow B cell depletion. However, in spite of preexisting immunity, they needed repeated exposures to the antigen, ie, similar to individuals who have not been vaccinated before.

Clinically, patients receiving anti-Y 29/55 depleted marrow were not more susceptible to infection and appeared to have fewer tumor relapses after ABMT as compared with recipients of untreated marrow. No patient has been lost due to infection. Five of the six patients with B cell non-Hodgkin’s lymphoma transplanted in first remission with anti-Y 29/55 depleted marrow became long-term survivors (>38 months; one patient succumbed to the tumor 3.5 months after ABMT). From all patients with the same neoplastic condition receiving untreated marrow four of eight were surviving (>56 months; three died from tumor relapse 2 to 7 months after ABMT and one from acute toxicity). Though these observations are promising, the relevance of this bone marrow decontamination procedure with regard to tumor control and patient survival cannot be judged conclusively from this small series. We conclude from this study that in vitro anti-Y 29/55 depletion of the bone marrow graft used for autologous transplantation does not cause defects of humoral immune function.

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