Host-mediated Antibody-dependent Cellular Cytotoxicity Contributes to the in Vivo Therapeutic Efficacy of an Anti-CD7-Saporin Immunotoxin in a Severe Combined Immunodeficient Mouse Model of Human T-Cell Acute Lymphoblastic Leukemia

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ABSTRACT

We have investigated the anti-leukemia effect that is exerted by the murine anti-CD7 antibody HB2 in a severe combined immunodeficient (SCID) mouse model of human T-cell acute lymphoblastic leukemia (T-ALL) and determined the contribution that this antibody effect makes to the therapeutic potency of a saporin immunotoxin (IT) constructed with the same antibody. The anti-leukemia effect is not exerted through complement-mediated lysis or through direct growth-inhibitory signaling after binding of antibody to the CD7 molecule on the T-ALL cell surface but rather through antibody-dependent cellular cytotoxicity (ADCC). Thus, the in vivo depletion of SCID mice of their natural killer cells almost completely abolishes the therapeutic effect of native HB2 anti-CD7 antibody and moreover significantly reduces the in vivo therapeutic performance of the anti-CD7 HB2-SAPORIN IT. Furthermore, an IT constructed with the F(ab')2 fragment of the same anti-CD7 antibody (HB2-F(ab')2-SAPORIN), which is incapable of recruiting natural killer cells, performed significantly less well therapeutically than HB2-SAPORIN IT. There was also a significant improvement in the therapeutic performance of the HB2-F(ab')2-SAPORIN IT in SCID-HSB-2 mice when used in combination with intact HB2 antibody, presumably through restoration of an ADCC attack on the target HSB-2 cell. These combined data indicate that ADCC in the SCID mouse does contribute additively together with toxin to the in vivo therapeutic potency of the HB2-SAPORIN IT directed against this human T-ALL cell line and that this has potentially important implications for the utility of this and other related classes of immunotherapeutic in human therapy.

INTRODUCTION

SCID3 mouse models of human leukemia and lymphoma have proved a useful tool for the preclinical evaluation of IT-based therapeutics. However, it is clear from many studies that native murine monoclonal antibody from which the immunoonjugate is constructed can exert an antitumor effect per se, evidenced by a prolongation in survival of antibody-treated animals compared with sham-treated controls (1–3). This antitumor effect is independent from the cytotoxicity that is exerted after toxin delivery, and the question therefore arises as to the contribution the antibody effect might make to the overall therapeutic outcome that is obtained with IT.

We have observed previously that the murine anti-human CD7 antibody HB2 exerts an anti-leukemia effect against the CD7+ human T-ALL cell line HSB-2 growing in CB.17 scid/scid mice (SCID-HSB-2 mice; Refs. 1 and 4). Although this antitumor effect is significant, it is however considerably weaker than that obtained with a saporin IT constructed with the same antibody. Although antibody treatment prolongs survival of HSB-2–bearing SCID mice, it does not generally lead to effective cures in the majority of animals, unlike IT, which does. This would imply that HB2 antibody either induces a state of tumor cell dormancy similar to that described by others (5) or alternatively, that antibody alone is only capable of a partial elimination of leukemia cells from the SCID host, with the surviving cells emerging sometime later after antibody treatment. SCID mice are profoundly deficient in T and B lymphocytes but still possess relatively intact NK cell and serum complement activities (6, 7). Any antitumor effect exerted by native murine antibody might therefore be mediated via one or both of these two potential mechanisms or alternatively through a direct signaling effect after binding of antibody. The work described here was undertaken to answer two important, related questions: (a) what is the in vivo mechanism of leukemia cell kill by HB2 antibody in SCID-HSB-2 mice? and (b) what contribution does this effect make, if any, to the overall therapeutic outcome that is obtained in human T-ALL (HSB-2) bearing SCID mice after therapy with a saporin IT constructed with the same murine anti-CD7 antibody?

MATERIALS AND METHODS

SCID Mice. Pathogen-free CB.17 scid/scid (SCID) mice of both sexes 6–10 weeks of age were produced from our own breeding colony maintained under British Government Home Office regulations and used in all of the experimental work described here. All manipulations on experimental animals were carried out in a laminar flow hood, and these animals were maintained in filter top microisolator cages and provided with sterile food and water ad libitum.

HSB-2 Human T-ALL Cell Line and YAC-1 Cell Line. The CD7+ human cell line HSB-2 was originally established from peripheral blood leukemic blasts from a 4-year-old pediatric patient with terminal T-ALL (8). The murine lymphoma cell line YAC-1 cell line, which is constitutively sensitive to NK cell-mediated lysis, has been described previously (9). Both cell lines were maintained in the logarithmic phase of growth in culture flasks containing antibiotic-free RPMI 1640 with 10% PCS and supplements of 2 mM sodium pyruvate and 2 mM glutamine (referred to as R10 medium) at 37°C under a humidified atmosphere of 5% CO2.

Antibodies and Antibody F(ab')2 Fragments. The rat anti-mouse antibodies AT37 (anti-CD2), 6D5 (anti-CD19), and F4/80 (anti-macrophage) and the mouse anti-mouse NK cell antibody NK1.1 directly conjugated to phycoerythrin were obtained from Serotec (Kidlington, UK). The rat antibody 2.4G2 directed against mouse FcγRII/III (anti-CD16/32) was obtained from PharMingen (San Diego, CA).

The anti-human CD7 antibody producing murine hybridoma clone HB2 was originally obtained from the American Tissue Culture Collection (Bethesda, MD). Bulk anti-CD7 antibody was produced on a Cellex AcuSyst R hollow fiber bioreactor system (Cellex, Minneapolis, MN). Antibody was purified by DEAE Sepharose ion exchange chromatography and gave a single band of Mr 160,000 on SDS-PAGE analysis under nonreducing conditions and retained full immunoreactivity as demonstrated by flow cytometry. F(ab')2 fragments of HB2 antibody were produced by papain digestion of native HB2 antibody using an ImmunoPure F(ab')2 kit from Pierce (Rockford, IL) following the manufacturer’s instructions. F(ab')2 HB2 antibody produced in this way gave a single band of Mr 110,000 on SDS-PAGE analysis under nonreducing conditions and appeared to be wholly free of contaminating Fc.

Saporin Purification. Seeds of Saponaria officinalis were kindly supplied by Chiltern Seeds (Ulverston, Cumbria, United Kingdom). The SO6 isofrom of Saporin Purification.

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3The abbreviations used are: SCID, severe combined immunodeficient; NK, natural killer; IT, immunotoxin; ADCC, antibody-dependent cellular cytotoxicity.
the ribosome inactivating protein saporin was extracted and purified to homogeneity from seeds as described previously (10).

**IT Construction.** The ITs HB2-SAPORIN and HB2-F(ab')2-SAPORIN ITs to HSB-2 cells were constructed by conjugating HB2 antibody or its F(ab')2 fragment to saporin using the heterobifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithio)propionate as described previously (11). Only ITs containing two saporin moieties per IT molecule were used in these studies because of their well-defined characteristics and potency as described by us previously (12). The purity of ITs was confirmed by SDS-PAGE and were then dialysed into PBS (pH 7.2), sterilized by passage through a 0.2 µm filter, and stored deep frozen in 100-µg aliquots at −80°C.

**Binding of HB2 Antibody and ITs to HSB-2 Cells.** The binding of HB2-SAPORIN and HB2-F(ab')2-SAPORIN ITs to HSB-2 cells was confirmed and compared with that obtained for the intact native HB2 antibody or its F(ab')2 fragment by flow cytometry. One million HSB-2 cells were incubated in the presence of 0.1% sodium azide for 30 min at 4°C with a saturating molar concentration (62.5 nM) of each IT or native HB2 antibody or its F(ab')2 fragment diluted in PBS (pH 7.2) containing 0.1% sodium azide. The negative controls comprised of HSB-2 cells were incubated in exactly the same way with an irrelevant, isotype-matched control antibody (BU-12, anti-CD19) at the same concentration. Cells were washed twice in cold PBS containing 0.1% sodium azide, and the cell pellets were incubated for an additional 30 min in 50 µl of FITC-labeled F(ab')2 rabbit anti-mouse immunoglobulins (Sigma Chemical Co., Poole, United Kingdom) diluted 1:50 in PBS. Cells were washed twice in cold PBS containing azide and resuspended in cold PBS: surface fluorescence was quantified on a Coulter Epics XL flow cytometer equipped with XL analytical software. In competitive inhibition studies, one million HSB-2 cells were incubated for 30 min at 4°C with FITC-labeled HB2 antibody at a concentration of 25 µM, together with varying concentrations of intact HB2 antibody, the F(ab')2 fragment of HB2 antibody, and HB2-SAPORIN IT or HB2-F(ab')2-SAPORIN IT as a competitor in the presence of 0.1% sodium azide. Cells were washed twice in cold PBS containing azide, and surface fluorescence was quantified by flow cytometry.

**Chromium Release Assay.** A chromium release assay was used to assess the constitutive lytic capabilities of SCID mouse splenocytes for HSB-2 and YAC-1 cells in the absence of HB2 antibody or in the presence of increasing amounts of intact or F(ab')2 HB2 antibody in an ADCC assay. Briefly, SCID mice were injected i.v. with 100 µg of polynucleosinopoly CMP (Sigma Chemical Co., Poole, United Kingdom) diluted 1:50 in PBS. Cells were washed twice in cold PBS containing azide and resuspended in cold PBS: surface fluorescence was quantified on a Coulter Epics XL flow cytometer equipped with XL analytical software. In competitive inhibition studies, one million HSB-2 cells were incubated for 30 min at 4°C with FITC-labeled HB2 antibody at a concentration of 25 µM, together with varying concentrations of intact HB2 antibody, the F(ab')2 fragment of HB2 antibody, and HB2-SAPORIN IT or HB2-F(ab')2-SAPORIN IT as a competitor in the presence of 0.1% sodium azide. Cells were washed twice in cold PBS containing azide, and surface fluorescence was quantified by flow cytometry.

**Protein Synthesis Inhibition Assay.** The inhibition of each IT and HB2 antibody to inhibit protein synthesis in target HSB-2 cells was evaluated by a 3[H]leucine incorporation assay as described previously (13).

**Chromium Release Assay.** A chromium release assay was used to assess the constitutive lytic capabilities of SCID mouse splenocytes for HSB-2 and YAC-1 cells in the absence of HB2 antibody or in the presence of increasing amounts of intact or F(ab')2 HB2 antibody in an ADCC assay. Briefly, SCID mice were injected i.v. with 100 µg of polynucleosinopoly CMP (Sigma Chemical Co., Poole, United Kingdom) diluted 1:50 in PBS. Cells were washed twice in cold PBS containing azide and resuspended in cold PBS: surface fluorescence was quantified on a Coulter Epics XL flow cytometer equipped with XL analytical software. In competitive inhibition studies, one million HSB-2 cells were incubated for 30 min at 4°C with FITC-labeled HB2 antibody at a concentration of 25 µM, together with varying concentrations of intact HB2 antibody, the F(ab')2 fragment of HB2 antibody, and HB2-SAPORIN IT or HB2-F(ab')2-SAPORIN IT as a competitor in the presence of 0.1% sodium azide. Cells were washed twice in cold PBS containing azide, and surface fluorescence was quantified by flow cytometry.

**RESULTS**

**Binding Characteristics of Intact and F(ab')2 Antibody and IT to HSB-2 Cells.** The fluorescence intensity of HSB-2 cells stained indirectly with either F(ab')2 antibody or with a saporin IT constructed with F(ab')2 antibody was approximately one-half of that seen for the respective intact antibody equivalents at a saturating equimolar concentration of 62.5 nM (Table 1). The fluorescence intensities achieved by ITs constructed with either intact or F(ab')2 antibody were also lower than those achieved by the respective antibody or F(ab')2 antibody fragment (Table 1).

The results showing competitive inhibition by intact HB2 antibody of the binding of molar equivalent amounts of intact and F(ab')2 antibody and ITs constructed with these to HSB-2 cells are shown in Fig. 1A and B, respectively. Fig. 1A reveals that the intact and F(ab')2 antibodies competed in a very similar manner with only a very minor difference between them. ITs constructed with intact HB2 and F(ab')2 antibody also competed in a very similar manner, although at the highest concentration of HB2 antibody as competitor (10−6 M), a clear although small difference did emerge between the two, with HB2-F(ab')2-SAPORIN IT more readily competed for by intact HB2 antibody (Fig. 1B).

**Protein Synthesis Inhibition in HSB-2 Cells by Intact and F(ab')2 Antibodies and ITs.** The protein synthesis inhibitory effects of HB2-SAPORIN IT, HB2-F(ab')2-SAPORIN IT, intact HB2 antibody, and native saporin on target HSB-2 cells are shown in Fig. 2. HB2-SAPORIN IT constructed with intact HB2 antibody achieved its IC50 at a concentration of 1 × 10−11 M compared with 3.8 × 10−11 M for the HB2-F(ab')2-SAPORIN IT, giving an almost 4-fold difference.

| Table 1 Mean fluorescence intensity of HSB-2 cells on flow cytometry after indirect staining with 6.25 nM intact HB2 or F(ab')2 HB2 antibody, HB2-SAPORIN IT, or HB2-F(ab')2-SAPORIN IT |
|-----------------|-----------------|-----------------|
| Reagent         | Mean fluorescence intensity (±SD) |
| HB2 Ab          | 45.85 ± 1.75    |
| HB2-F(ab')2     | 21.60 ± 1.10    |
| HB2-SAPORIN IT  | 35.85 ± 0.13    |
| HB2-F(ab')2-SAPORIN IT | 14.65 ± 0.25 |

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Plates were incubated overnight (14−16 h) at 37°C in a humidified 5% CO2 atmosphere, and 100 µl of supernatant were carefully removed from each well for counting on a gamma counter. The amount of specific target cell lysis expressed as a percentage was calculated from the total amount of radioactivity released after NP40 detergent lysis of untreated HSB-2 target cells compared with the amount released after treatment with antibody or antibody plus complement at the respective concentrations studied.

**SCID Mouse Experiments.** On day one of the study, groups of 10 SCID mice were injected with two million HSB-2 cells via the tail vein in a 200-µl volume of R10 medium. The growth and dissemination of HSB-2 human T-ALL in SCID mice has been described in detail by us previously (1). Treatment with a 6.25 nM amount of intact or F(ab')2 HB2 antibody, HB2-SAPORIN, or HB2-F(ab')2-SAPORIN in a 200-µl volume of PBS was given as a single i.v. bolus injection into the tail vein on day 7. Groups of 10 control animals were sham treated with 200 µl of PBS also on day 7. Experiments were also conducted in which SCID mice had been depleted of functional NK cells by prior treatment with rabbit anti-asialo G1m, antiserum. Here an i.v. injection of 20 µl of rabbit anti-asialo G1m, antiserum (14, 15) in 180 µl of PBS (Wako Chemicals GmbH, Neuss, Germany) was given 6 days after injection of HSB-2 cells and was followed by treatment with antibody or IT as usual on day 7. Animals were monitored twice daily, and those showing distress or obvious signs of illness were humanely killed and subjected to postmortem examination to confirm the presence (or absence) of disease.

**Statistical Analysis.** Survival analysis was carried out by log-rank analysis (Peto’s method) using the SOLO statistics software package (BMDP Statistical Software, Los Angeles, CA). P values of 0.05 or less were considered as statistically significant in these studies.
NK Cell Activity of SCID Mouse Splenocytes for YAC-1 and HSB-2 Cells. Constitutive NK cell-like activity of SCID splenocytes used over a range of E:T ratios for YAC-1 and HSB-2 target cells are shown in Fig. 3. Splenocytes from SCID mice treated 24 h previously with polyinosinic-polyCMP were capable of causing between 40 and 50% specific lysis of target YAC-1 cells over all E:T target ratios studied. In contrast, SCID mouse splenocytes did not cause lysis of HSB-2 cells, demonstrating the relative resistance of this human T-ALL cell line to NK cell-mediated lysis (Fig. 3).

HB2 Antibody-mediated ADCC of SCID Mouse Splenocytes for HSB-2 Cells. Results obtained for the specific lysis of target HSB-2 cells in an ADCC assay with native HB2 antibody, a F(ab')2 fragment of HB2 antibody, HB2-SAPORIN IT constructed with native HB2 antibody, or the irrelevant, isotype-matched anti-CD19 antibody BU12 using SCID splenocytes as effector cells at an E:T ratio of 100:1 are shown in Fig. 4. We observed an antibody dose-dependent increase in HSB-2 target cell lysis that plateaued at between 36 and 42% specific target cell lysis at HB2 antibody or HB2-SAPORIN IT concentrations of between 62.5 and 625 nM (Fig. 4). The F(ab')2 fragment of HB2 antibody used in the assay over the same molar

ence in potency between these two ITs. Native saporin achieved its IC50 at a concentration of 2 × 10⁻⁸ M, making the HB2-SAPORIN IT and HB2-F(ab')2-SAPORIN ITs 20,000-fold and 5,263-fold more potent than saporin, respectively. Native HB2 antibody had no significant effect on HSB-2 protein synthesis levels.

Antiproliferative Effects of HB2-SAPORIN IT and Intact HB2 Antibody on HSB-2 Cells. The effects of HB2-SAPORIN IT and native intact HB2 antibody at an equimolar concentration of 6.25 nM on the proliferation of HSB-2 cells in flask culture was studied. HB2-SAPORIN IT completely inhibited HSB-2 proliferation for the 50-day duration of the study, whereas HB2 antibody had absolutely no effect on HSB-2 proliferation, which occurred at the same rate as in untreated control cultures (data not shown).

Immunophenotypic Analysis of SCID Mouse Splenocytes. Flow cytometry was conducted on SCID mouse splenocytes to determine the relative proportions of T and B lymphocytes, NK cells, and macrophages present. Clearly, there was a paucity of B and T lymphocytes within the splenic cell population with only 2% staining with an anti-CD19 (B-cell) and 4.65% with an anti-CD2 (T-cell) antibody. Thirty-two % of SCID mouse splenic cells stained with the NK cell antibody NK1.1, and 34% stained with the CD16/CD32 antibody, identifying both FcyRII and FcyRIII. Just over 16% of splenic cells stained positively with the antibody F4/80, which recognizes macrophages.
concentration range did not result in any significant HSB-2 target cell lysis. Similarly, the irrelevant intact anti-CD19 antibody BU12 when used in the assay did not result in any HSB-2 target cell lysis (Fig. 4).

We also studied the effects of depleting SCID mouse splenocytes of their adherent cell population on their resultant ADCC activity for HB2 antibody-coated HSB-2 cells. Passing SCID splenocytes through a glass bead column as described in "Materials and Methods" led to a reduction in the number of macrophages staining with F4/80 antibody by flow cytometry from 16 to <3%, whereas the relative proportion of NK1.1+ NK cells was enriched from 33 to 52%. Comparative analysis of the performance of untreated SCID splenocytes versus adherent cell-depleted SCID splenocytes in ADCC is shown in Fig. 5. Depletion of the adherent cell population led to a significant increase in antibody dose-dependent ADCC activity of depleted splenocytes against HSB-2 cells with a greater than doubling of lytic activity at the two highest antibody concentrations used. Untreated or adherent cell-depleted SCID mouse splenocytes had no lytic activity for HSB-2 cells when the irrelevant anti-CD19 IgG1 control antibody BU12 was used over the same concentration range (Fig. 5).

We also investigated the effect of administering a single i.v. injection of rabbit anti-asialo GM1 antibody to SCID mice on the lytic capacity of splenocytes taken from these animals against HSB-2 cells in an ADCC assay with native HB2 antibody. The results of this study, shown in Fig. 6, clearly show that treatment with anti-asialo GM1 antibody significantly reduced the ability of splenocytes from these animals to bring about HSB-2 target cell lysis in this ADCC assay. Thus, treatment of target HSB-2 cells at the highest HB2 antibody concentration used (0.625 nm) with splenocytes taken from anti-asialo GM1 antibody-treated animals resulted in only 10% specific lysis compared with 66% achieved with splenocytes from untreated animals.

Complement-mediated Lysis of HSB-2 Cells. There was no significant observable lysis of HSB-2 cells after incubation with increasing concentrations of HB2 antibody in the presence of 5% fresh SCID mouse serum (data not shown).

Survival of SCID-HSB-2 Mice Treated with Intact or F(ab')2 Antibodies and Immunotoxins. The survival of SCID-HSB-2 mice treated with a single i.v. injection of 6.25 nmol of intact HB2 antibody, F(ab')2 HB2 antibody, BU12 intact antibody (anti-CD19), or sham-treated with a single injection of PBS is shown in Fig. 7A. Animals treated with F(ab')2 HB2 antibody or with intact BU12 antibody survived for a similar period of time as PBS sham-treated controls with mean survival times of 61, 61, and 53 days for each of these groups, respectively, there being no significant differences between them. In contrast, animals treated with intact HB2 antibody showed a significant (P = 0.0056) prolongation in survival compared with PBS controls (mean survival, 97 days versus 53 days), with the last animal in this group dying with disease at 163 days. The survival of animals treated with HB2-SAPORIN IT, HB2-F(ab')2 SAPORIN IT, or with PBS is shown in Fig. 7B. In this particular experiment, treatment with HB2-SAPORIN gave a 40% survival rate and a mean survival time of 118 days compared with only a 20% survival rate and 88-day mean survival time for HB2-F(ab')2-SAPORIN treated animals. It is of interest and of potential importance to note that one-half of the animals treated with HB2-F(ab')2-SAPORIN died early and at a similar rate to the PBS controls.
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Fig. 8A shows the survival of NK cell-intact and NK cell-depleted SCID-HSB-2 mice treated with HB2-SAPORIN IT compared with the survival of PBS sham-treated control animals. In this particular study, all PBS sham-treated controls were dead by day 78, with a mean survival time for this group of 51 days. The therapeutic effect of HB2-SAPORIN IT was markedly better in SCID-HSB-2 mice in which NK cell activity was intact. Thus, NK cell-depleted SCID-HSB-2 mice treated with HB2-SAPORIN IT had a mean survival time of 71 days, with 40% survivors upon termination of the study at 108 days (P = 0.145), whereas in comparison, NK cell-intact SCID-HSB-2 mice had a mean survival time of 97 days, with 80% survivors at 108 days upon termination of the experiment (P = 0.000736). Sixty % of the NK cell-depleted animals that were treated with HB2-SAPORIN IT died early and at the same rate as the PBS sham-treated controls, and it is this reason that accounted for the lack of statistical significance between this therapy group and PBS controls, although 40% of the animals survived disease free.

Partial Restoration of the in Vivo Therapeutic Potency of HB2-F(ab')2-SAPORIN with Intact HB2 Antibody. We conducted an experiment to establish whether the in vivo therapeutic potency of the HB2-F(ab')2-SAPORIN IT could be improved to a level comparable with that seen with HB2-SAPORIN IT constructed with intact HB2 antibody by treating SCID-HSB-2 mice with a combination of HB2-

It was the occurrence of these early deaths that accounted for the lack of statistical significance that was found following log-rank analysis comparing the PBS sham-treated animals with the HB2-F(ab')2-SAPORIN (P = 0.0622). The HB2-SAPORIN IT-treated animals, on the other hand, did show a statistically significant therapeutic effect when compared with PBS sham-treated controls (P = 0.00486). When the HB2-SAPORIN and HB2-F(ab')2-SAPORIN therapy groups are compared with each other by log-rank analysis, they were found to be significantly different from one another (P = 0.0422).

Therapeutic Performance of Intact HB2 Antibody and HB2-SAPORIN IT in NK Cell-depleted SCID Mice. The results of intact HB2 antibody treatment of SCID-HSB-2 mice depleted of their NK cells in comparison to SCID-HSB-2 mice possessing intact NK cells are shown in Fig. 8A. There was no significant difference between the PBS sham-treated control group and the group depleted of their NK cells by anti-asialo Gm1 antibody treatment, with mean survival times of 43 and 38 days for each of these groups, respectively. Treatment of NK cell intact SCID-HSB-2 mice with intact HB2 antibody led to a highly significant prolongation in survival when compared with the PBS sham control group (mean survival of 72 days versus 43 days, respectively; P = 0.0097). Treatment of NK cell-depleted SCID-HSB-2 mice with intact HB2 antibody resulted in only a modest prolongation in mean survival time (51 days versus 38 days for the anti-asialo Gm1 antibody-treated control group), which was just significant when compared with the NK cell-depleted control animals (P = 0.0481) but not when compared with the NK cell-intact PBS sham-treated control animals (P = 0.328).

Fig. 8B shows the effects of depletion of SCID-HSB-2 mice of functional NK cells with anti-asialo Gm1 antibody on their survival after treatment with antibody or IT. Animals were injected i.v. with two million HSB-2 cells on day 1, followed by treatment as described in "Materials and Methods" with: A, anti-asialo Gm1 antibody on day 6 and native HB2 antibody on day 7 (O); PBS sham-treated on day 7 (1), or B, anti-asialo Gm1 antibody on day 6 and HB2-SAPORIN IT on day 7 (A), PBS sham-treated on day 6 (1), or PBS sham-treated on days 6 and 7 (1).
F(ab')2-SAPORIN and intact HB2 antibody. In this study, groups of animals were treated i.v. with a single 6.25 nm dose of HB2-SAPORIN, HB2-F(ab')2-SAPORIN, or an equimolar combination of both HB2-F(ab')2-SAPORIN + intact HB2 antibody. The survival of animals in the various treatment groups is shown in Fig. 9. As demonstrated previously, the HB2-F(ab')2-SAPORIN IT was significantly less effective than HB2-SAPORIN constructed with intact HB2 antibody (P = 0.0345), with mean survival times of 53 and 79 days and 20 and 80% survivors in these groups, respectively. The addition of intact HB2 antibody significantly improved the therapeutic performance of HB2-F(ab')2-SAPORIN IT, increasing the observed mean survival time from 53 to 69 days and improving the survival rate from 20 to 60%. Log-Rank analysis revealed that although both HB2-SAPORIN and HB2-F(ab')2-SAPORIN ITs had significant therapeutic effects compared with PBS sham-treated animals (P = 0.001268 and 0.04441, respectively), they were also significantly different from each other in therapeutic performance (P = 0.0345). There was no significant difference between the therapeutic performance of HB2-SAPORIN IT constructed with intact HB2 antibody and HB2-F(ab')2-SAPORIN IT plus intact HB2 antibody (P = 0.3047).

**DISCUSSION**

This work was conducted with two objectives in mind: (a) to elucidate the mechanism by which the murine anti-human CD7 antibody HB2 exerts its anti-leukemia effect in the SCID-HSB-2 model of human T-ALL; and (b) to establish whether this antibody-mediated, anti-leukemia effect contributes in any way to the therapeutic effectiveness of HB2-SAPORIN IT in the SCID-HSB-2 model. The in vitro data presented here indicate that HB2 antibody has no direct effect on either protein synthesis or on the proliferative capacity of HSB-2 cells, and we therefore conclude that direct cell signaling by antibody is unlikely to play any role in the observed anti-leukemia effect that occurs in vivo. This contrasts sharply with recent findings that have shown that anti-CD19 antibodies exert at least some of their anti-human lymphoma effects in SCID mice via direct signal transduction after antibody binding to CD19 on the tumor cell surface, which leads to cell cycle arrest (16).

The anti-leukemia effect exerted by HB2 antibody in SCID-HSB-2 mice is therefore likely mediated via recruitment of host humoral or cellular effectors. The observation that native HB2 antibody exerts a significant anti-HSB-2 leukemia effect in vivo, whereas a F(ab')2 derivative of the same antibody does not, demonstrates the importance of the Fc domain of this antibody to the antitumor mechanism that is operative. HB2 antibody is an IgG1 and is therefore capable of fixing complement and of recruiting cytotoxic host cellular effectors bearing IgG Fc receptors (FcγR) on their surface. However, the observation that no significant HSB-2 cell lysis occurred in vitro in the presence of HB2 antibody plus 5% fresh SCID mouse serum leads us to conclude that complement-mediated lysis of HSB-2 cells is not the likely mechanism of leukemia cell growth delay in vivo.

The substantially reduced fluorescence intensities observed for both the F(ab')2 HB2 antibody fragment and for the HB2-F(ab')2-SAPORIN IT compared with their respective intact antibody counterparts might at first be interpreted as a demonstration of reduced binding capacity of these reagents for CD7 on the cell surface. However, competitive inhibition of a direct HB2-FITC antibody conjugate by the F(ab')2 derivatives did not reveal any very major differences in antibody binding capacity between the intact and F(ab')2 derivatives, as suggested by the original indirect method. The interpretation here is clear; the removal of the Fc domain in the F(ab')2 reagents eliminates epitopes seen by the polyclonal goat anti-mouse immunoglobulin-FITC secondary reagent that would normally be present in the intact antibody. As a consequence, fewer secondary FITC-labeled antibodies are able to bind to the F(ab')2 derivatives, resulting in a lower fluorescent signal.

Our data suggest that the mechanism of HSB-2 cell elimination in vivo is probably via ADCC subsequent to the recruitment of FcγR-bearing cytotoxic SCID mouse effector cells. Although SCID mice are profoundly deficient in both T and B lymphocytes (17), they do, however, possess fully functional NK cells and tissue macrophages (6). In this regard, we were able to demonstrate that SCID mouse splenocytes were constitutively cytotoxic in vitro for target YAC-1 cells but not for HSB-2 cells. Decreasing the number of SCID mouse effector cells over the range studied did not result in decreased cell lysis of target YAC-1 cells, and this possibly reflects the relatively high proportion of SCID splenocytes that are actually NK cells (32% compared with <5% in immunocompetent mice), resulting in an apparent increase in efficiency of target cell killing. SCID mouse splenocytes were, however, capable of lysing HSB-2 cells when intact anti-CD7 antibody HB2 was bound to the surface of the cell in an antibody dose-dependent manner. A F(ab')2 preparation of the same HB2 antibody was incapable of lysing HSB-2 cells in the ADCC assay, indicating that lysis was mediated via the Fc domain of antibody, probably via engagement with Fc receptors expressed by NK cells or macrophages. An ADCC mechanism is further supported by the observation that the therapeutic effect of HB2 antibody in the SCID-HSB-2 model is completely lost in animals that have been depleted of their NK cells.

**IMMUNOPHENOTYPIC ANALYSIS OF SCID MOUSE SPLENIC CELL POPULATIONS**

The depletion of F4/80 splenocyte populations was essentially complete in 80% of mice, whereas 90% of animals demonstrated a >5% decrease in Thy-1+ T-cell numbers, reflecting the loss of allogeneic T cells in the SCID-HSB-2 model. The average percentage of F4/80+ macrophage depletion was 90%, with a range of 80% to 95% as compared with 20% in immunocompetent mice. The depletion of NK1.1+ NK cells was <5%, whereas the depletion of Thy-1+ T cells was >95% in all mice. The depletion of F4/80+ macrophages and Thy-1+ T cells was confirmed by flow cytometry and by immunohistochemical analysis of splenic sections. The depletion of NK1.1+ NK cells was not as pronounced as that of F4/80+ macrophages and Thy-1+ T cells, with a range of 70% to 90% as compared with <5% in immunocompetent mice. The depletion of NK1.1+ NK cells was confirmed by flow cytometry and by immunohistochemical analysis of splenic sections. The depletion of Thy-1+ T cells was repeatable, with an average depletion of 90%, with a range of 80% to 95% as compared with 20% in immunocompetent mice. The depletion of NK1.1+ NK cells was not as pronounced as that of F4/80+ macrophages and Thy-1+ T cells, with a range of 70% to 90% as compared with <5% in immunocompetent mice. The depletion of Thy-1+ T cells was confirmed by flow cytometry and by immunohistochemical analysis of splenic sections.
G~M~1~ antibody (which selectively damages NK cells) had a significantly reduced in vitro lytic capacity for HSB-2 cells in the ADCC assay really points to the NK cell as being the major effector in this system. Indeed, removal of macrophages from the splenic cell population led, in this study, to an increased lytic capability of the remaining splenic cells for HB2 antibody-coated HSB-2 cells. This might indicate one of two things, either that macrophages exert an inhibitory effect on NK cell-mediated ADCC or that their removal allows access of proportionately greater numbers of lytically more efficient NK cells to the target HSB-2 cell.

What contribution, therefore, if any, does any ADCC response directed against antibody-coated HSB-2 cells make to the overall therapeutic outcome that is obtained in vivo in SCID-HSB-2 mice treated with HB2-SAPORIN IT? We clearly demonstrated that the HB2-SAPORIN IT constructed with intact HB2 antibody was virtually as effective as native HB2 antibody at lysing target HSB-2 cells in vitro, and it should therefore be as capable as antibody at participating in ADCC in vivo. Does ADCC, therefore, combined with IT-mediated toxin delivery to target HSB-2 cells, work in concert to achieve an additive or more than additive therapeutic effect? There are three separate pieces of experimental evidence presented here that suggest that this is indeed the case: (a) the therapeutic effectiveness of HB2-SAPORIN constructed with intact HB2 antibody is significantly better than that of HB2-F(ab')2-SAPORIN, which is incapable of recruiting NK cells; (b) NK cell depletion of SCID-HSB-2 mice resulted in a significant reduction in the therapeutic effectiveness of both the intact HB2 antibody and HB2-SAPORIN IT constructed with intact HB2 antibody; and (c) the therapeutic effectiveness of HB2-F(ab')2-SAPORIN in SCID-HSB-2 mice is significantly improved by the addition of intact HB2 antibody, presumably through the restoration of an ADCC membrane attack on the HSB-2 cell via the intact antibody bound to the cell surface. This is, however, a potential conflict of interest between the two different mechanisms of cell kill. In the case of IT, internalization of the IT to the appropriate cellular compartment is a prerequisite for cell killing, whereas in the case of ADCC, internalization would be a positive disadvantage, the requirement being for display of target-bound antibody on the cell surface for engagement with cytotoxic effector cell FcγR. The dual attack system described here is, therefore, a compromise and may be functioning suboptimally. Thus, alternative strategies may need to be sought to optimize each mechanism in relation to the other, one realistic possibility being to use a second antibody recognizing a completely unrelated, non-internalizing, coexpressed surface antigen on the target and thus deliver the ADCC attack in this way.

The in vitro potency of HB2-F(ab')2-SAPORIN was shown to be reduced 3-fold in comparison to HB2-SAPORIN, a finding in keeping with the previous observations of others. This reduction in potency may possibly be ascribed to the small decrease in the binding capacity of the F(ab')2 IT, an observation again supported by similar observations made by others and which in our opinion possibly reflects some partial steric hindrance of the antigen binding site due to the saporin moiety being attached closer to the antibody hypervariable region. To support this contention, we did find by flow cytometry a small though real reduction in binding capacity of the F(ab')2 IT compared with the intact antibody IT construct. It might be reasonably argued that the small observed reduction in the in vitro effectiveness of the HB2-F(ab')2-SAPORIN IT might have accounted for its reduced in vivo therapeutic effectiveness compared with HB2-SAPORIN in the SCID-HSB-2 model, and this is undoubtedly a valid criticism. If we were using only this single experimental observation to draw our final conclusions on the contribution of ADCC to IT potency, then this would obviously be unreliable and thus flawed, but the fact that we have presented three totally separate pieces of corroborating experimental evidence substantially strengthens this hypothesis.

It has been assumed by several groups, who have used SCID mouse models of human leukemia or lymphoma to establish the therapeutic efficacy of immunotoxin-based therapies, that the observed therapeutic effects have been solely due to toxin-mediated killing of target cells. Our study now suggests that this may not necessarily always be the case. Initial evidence that a host immune effector mechanism(s) might act additively with an IT to achieve an improved therapeutic outcome was provided by Shen et al. (18). These workers demonstrated in adoptive cell transfer studies in SCID mice bearing the human B-cell lymphoma cell line Daudi that a bispecific anti-CD22/anti-CD3 ricin A chain IT capable of recruiting LAK T cells via the anti-CD3 arm and of delivering toxin to Daudi targets via the anti-CD22 arm was more effective when used in conjunction with LAK T cells. Although this is a completely different system involving a totally different cytotoxic effector cell population, it does provide further independent evidence that host immune effector mechanisms can act in concert with toxin delivery to result in an additive therapeutic effect. There is, however, a possible alternative explanation that might account for the improved in vivo therapeutic performance of HB2-SAPORIN IT over HB2-F(ab')2-SAPORIN IT in SCID-HSB-2 mice. Van Oosterhout et al. (19) showed that FcγRII receptors on CD19+ target B cells enhanced the uptake and subsequent potency of an IgG1 subclass anti-CD19-RTA IT. An IT constructed with an IgG2a isotype switch variant of the same antibody that was incapable of binding to FcγRII through its Fc domain was very poorly cytotoxic. Vervoordeldonk et al. (20) further showed that cross-linking of CD19 to FcγRII on the Daudi cell line surface via the same IgG1 anti-CD19 antibody led to an increased rate of internalization of CD19. This did not occur with the IgG2a isotype switch variant. However, it is presently not known whether the effect described by van Oosterhout is restricted to only CD19 or is more widely shared by other surface molecules such as CD7. HSB-2 cells do not constitutively express FcγRII; therefore, the modest decrease in in vitro potency of HB2-F(ab')2-SAPORIN compared with HB2-SAPORIN could not have been due to such an effect. It is, however, conceivable that a similar effect may have been operating in vivo in our SCID-HSB-2 model via FcγRII-expressing SCID effector cells that may have led to an increased degree of IT internalization and subsequent improvement to the apparent therapeutic outcome. We feel, however, that this is a rather unlikely explanation for the data presented here, but nonetheless, further investigations are warranted to exclude this as a possibility.

If there is genuine additivity between toxin delivery and ADCC in this particular model system, as we think is more likely the case, then there are a number of implications that arise from these findings. Most important of these is that the utility of an immunotoxin constructed with a murine monoclonal antibody in human subjects will recruit human host cellular effectors only very poorly because of the relative inefficiency with which human Fc receptors expressed by human effector cells bind to murine Fc. It should also be kept in mind that the described results are in a murine system and may not be applicable to humans. We have, however, demonstrated that HB2 antibody-coated HSB-2 cells can be lysed in vitro by human peripheral blood mononuclear cells. If it finally emerges that ADCC does contribute to in vivo IT therapeutic potency as a general rule (and not just in the context of these limited findings in one specific model system), then IT's constructed with murine antibodies would likely perform suboptimally in relation to their full potential. Conferment of more efficient

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4 S. Warnes and D. J. Flavell, unpublished observations.
host effector recruitment machinery on the IT through the addition of a human Fc domain or domains (21) would be one possible strategy to ensure effective recruitment of human cytotoxic effectors. The idea of a focused multimodal attack on the tumor cell is not new, but relevant in vivo experimental evidence to support such a notion has previously been very limited. This study has provided potentially important preclinical evidence that justifies further investigation of this effect and provides the rationale for developing this concept and moving it forward into the design of new immunotherapeutic strategies for use in clinical studies in humans.

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