EMERGENCE OF IMMUNOGLOBULIN VARIANTS
FOLLOWING TREATMENT OF A B CELL LEUKEMIA WITH
AN IMMUNOTOXIN COMPOSED OF ANTIIDIOTYPIC
ANTIBODY AND SAPORIN

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Although monoclonal technology has done much to overcome the technical problems of providing antibodies for the treatment of neoplastic disease, the therapeutic effectiveness of antibodies in patients has been limited (1–3). However, during this work several significant factors have been identified that may thwart antibody attack on tumor cells. Prominent among these are: (a) the presence of extracellular antigen that can block antibody access to tumor cells; (b) the loss of antibody from the target cell surface due to antigenic modulation; (c) the apparent inefficiency with which natural effector mechanisms operate with infused antibody; and (d) the production of an antiantibody response in the patients.

A further problem, and one that is likely to become more prominent as the potency of antibody treatment increases, is that of tumor heterogeneity. Any variant tumor cell that can resist antibody attack, either because it lacks the target antigen or is not susceptible to lysis by natural effectors, could, under the selective pressure of antibody treatment, eventually emerge as the predominant phenotype. Until recently such immunoselection had been seen only in animals bearing tumors from long-established lines. Several groups demonstrated that antigen-negative cells present in these transplanted tumors can escape attack from therapeutic antibody (4, 5) or can survive in animals that have been preimmunized against a tumor-associated antigen (6, 7).

In clinical studies to date, tumors emerging in patients undergoing antibody treatment have either expressed the target antigen in an unaltered state, or been in a temporary state of antigenic modulation that reverted when the level of therapeutic antibody subsided. Recently, however, Meeker et al. (8) reported that 2 of 11 cases of human B cell lymphoma treated with monoclonal antiidiotype (anti-Id) later developed variant tumors that were unable to react with the

therapeutic antibody. The loss of reactivity appeared to stem from changes in the amino acid sequence of the Ig heavy chain variable (V) region as a result of point mutations in the gene (9). In a further study from the same group (10) multiple overlapping idiotype subpopulations were defined in two cases of follicular lymphoma before they had received any anti-Id treatment. Southern blot analysis again suggested extensive somatic mutation in heavy chain genes. An earlier report from Raffeld et al. (11) also found idiotypic variation in a human lymphoma before any anti-Id treatment: here a variant subclone emerged, unable to bind its anti-Id, after a transient spontaneous regression in the tumor. Thus, although human B cell neoplasia may be monoclonal by conventional criteria, they can still display variation in the structure of their Ig idiotypes.

Point mutations within the rearranged Ig V region occur as part of the normal process that helps generate antibody diversity in the B cell repertoire (12). Mutation frequencies within the V region DNA of responding B cells have been estimated to be as high as one basepair per 10^3 per cell division, several orders of magnitude above that quoted for many other eukaryotic genes (13, 14). It is also known that similar random mutations can accumulate in the rearranged Ig genes of certain myeloma and hybridoma cell lines (15). However these variants, unlike those in normal B cell clones, probably gain no selective advantage as a result of any structural changes in their Ig V regions, and consequently exist at comparatively low frequencies (10^{-3}–10^{-8}) within such populations (15, 16). It is now clear from the results discussed above that similar Ig variants can arise in human B cell lymphomas, and that they may gain a selective advantage when a change in their Id allows them to escape attack from anti-Id antibody. The frequency at which new Ig variants will arise in such tumor populations is not known, but the high mutational rate in normal B cells does not augur well for the use of a single idiotope as a target for antibody attack.

The L$_2$C lymphoblastic leukemia of strain 2 guinea pigs has proved a useful model for those human B cell neoplasms that are likely to be amenable to therapy with anti-Id. The major advantage of Ig Id as a target molecule is its tissue specificity, which for all practical purposes will be limited to the neoplastic B cell clone (17). One disadvantage has been that tumors under attack from anti-Id have been particularly prone to antigenic modulation, to the extent that they can become completely resistant to the cytotoxic actions of natural effectors in vitro and in vivo (3, 18). A possible solution to this problem would be to couple a toxin or similar molecule to the antibody to enable it to kill cells without the host's effector systems. Such immunotoxins have been prepared in many laboratories and have been shown to kill their target cells with great potency and specificity (19).

In the present work we have used an immunotoxin composed of monoclonal anti-Id and the ribosome-inactivating protein (RIP),[1] saporin, for the treatment of the L$_2$C tumor in guinea pigs. Saporin is one of a large group of single-chain RIPs found throughout the plant kingdom (20). They resemble the A chains of the plant toxins ricin and abrin in their ability to terminate protein synthesis but,

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[1] *Abbreviations used in this paper:* RIP, ribosome-inactivating protein; RN, receptor negative; SPDP, N-succinimidyl-3-(2 pyridyldithio)propionate.

unlike these toxins, do not have a B chain by which they can bind to and enter cells. The single-chain RIPs are therefore practically devoid of toxicity for intact cells. Conjugation to an antibody bestows on them the ability to bind and enter cells and shut down protein synthesis in much the same way as the A chain of the toxins (21–23).

We now show that the treatment of L_2C leukemia with anti-Id immunotoxins although highly effective at destroying tumor and protecting tumor-bearing animals, is usually thwarted by the emergence of variant tumors. These variants are of two types: (a) cells that are surface Ig–negative due to a loss of heavy chain production; (b) cells that express surface Ig, but with an altered Id that is no longer reactive with the therapeutic anti-Id.

Materials and Methods

Leukemic Cells. The L_2C lymphoblastic leukemia is maintained by in vivo passage in strain 2 guinea pigs and has been described previously (24). Cells exhibit monomeric surface IgMλ (50,000 molecules/cell), and in short-term culture secrete free λ light chain together with small amounts of idiotypic 19 S IgM (25). L_2C cells were isolated from fresh leukemic blood on Ficoll-Hypaque (Lymphoprep; Nyeguard, Oslo, Norway) and were always >98% viable by trypan-blue exclusion (26).

Production and Preparation of Antibodies. Polyclonal IgG antibody specific for mouse normal IgG was prepared and conjugated with fluorescein isothiocyanate isomer I (BDH Chemicals, Ltd., Poole, United Kingdom) (27). The FITC-labelled antibody showed no detectable reactivity with L_2C cells before they had been coated with mouse antibodies.

A panel of 16 mouse mAb, each specific for the L_2C IgMλ, has been used in this investigation. The production of these antibodies and analysis of their interaction with viable L_2C cells has been described by Elliott et al (27). For the present work we have retained the original numbering. 14 of the antibodies are specific for the Id of the L_2C IgM and were numbered sequentially, anti-Id-1–14, while two other antibodies, anti-λ and anti-μ, are directed against constant regions of the guinea pig λ light chain and μ heavy chain, respectively. The mAb anti-xId-9, although recognizing the L_2C Id, shows a limited crossreactivity for other guinea pig Ig (27, 28). A mouse IgG1 mAb (M15-8) showing no reactivity towards L_2C cells was used for all control studies (control IgG1).

An additional mAb, anti–free λ Id, was raised as described by Elliott et al. (27), using urinary λ chain isolated from L_2C leukemic guinea pigs as the immunogen (29, 30). In a standard ELISA (31), this antibody reacted strongly with L_2C urinary λ chains, weakly with guinea pig normal λ chain (29) and showed no reactivity with L_2C idiotypic 19 S IgM (32). Thus the epitope seen by anti–free λ Id is expressed on λ chain derived from the L_2C tumor, but is hidden or blocked in the intact IgM molecules. It appears also to be present on a minor population of guinea pig normal λ chains.

Monoclonal IgG was isolated from ascitic fluid that had been recovered from pristane-primed (BALB/c × CBA)F_1 mice carrying the appropriate hybridoma cells as an ascitic tumor. The IgG was first precipitated in 2 M ammonium sulfate in 0.2 M Trizma base (Sigma Chemical Co., St Louis, MO) and then chromatographed on Trisacryl-M-DEAE (LKB-Produkter AB, Bromma, Sweden), with a linear salt gradient of 5–50 mM phosphate, pH 8.0, at room temperature. The antibodies eluted in this way were of high purity by SDS-PAGE.

Immunofluorescence Studies. Binding of monoclonal antibodies to L_2C cells was analyzed by using the FACS III Becton-Dickinson Electronics, Mountain View, CA). 2 × 10⁷ cells/ ml in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate (Gibco Europe, Uxbridge, United Kingdom) were treated with mouse mAb or control IgG (5 μg/ml) for 30 min on ice, washed and exposed to fluorescent rabbit anti-mouse IgG at 0.5 mg/ml. After a final wash, cells were examined in the presence of sodium azide (10 mM), which was added to prevent any modulation of surface-bound antibodies.

Fluorescent antibody–labelled cells were sorted using the FACS III, and collected directly into a 1.5 ml plastic microfuge tube (Starstedt Laborartikel, Federal Republic of Germany) containing supplemented DMEM. Conditions for sorting were optimized using glutaraldehyde-fixed chicken erythrocytes at a flow rate of 1,000 cells/s and a drop-drive frequency of 37,000/s. Sorted cells were expanded by transfer into strain 2 guinea pigs (5×10^5 cells/animal).

Preparation of Antibody-saporin Immunotoxin. Saporin was purified from the seeds of *Saponaria officinalis* (soapwort) as described previously (33). It was coupled by disulfide linkage to mouse monoclonal IgG using *N*-succinimidyl-3-(2 pyridyldithio) propionate (SPDP). Conjugates eluting from gel filtration columns with an M_r corresponding to between 160,000 and 210,000 were used for the present work. They were found predominantly to contain one molecule of antibody and one or two molecules of saporin when analyzed as described by Thorpe et al. (23). The conjugate solutions were sterilized by filtration through 0.22-μm filter, and stored at $-70\,^\circ$C after first freezing in liquid nitrogen.

Uptake of [^{3}H]Leucine by L₂C Cells in Culture. Fresh L₂C cells 5×10^6 cells/ml in supplemented DMEM (see Immunofluorescence) were dispensed as 100-μl aliquots into 96-well flat-bottomed microplates (Gibco). Test samples of immunotoxin or diluted plasma in the same medium (100 μl/well) were then added and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 3 h, cells were pulsed with 0.5 μCi/well [^{3}H]leucine (TRK.510; Amersham International, Amersham, United Kingdom) in supplemented DMEM for a further 6 h at 37°C in 5% CO_2. The incorporation of [^{3}H]leucine into L₂C cells was assessed by harvesting the cells onto glass microfiber filters using an automatic cell harvester (Dynatech, Billingshurst, United Kingdom). The radioactivity retained on the filters after washing with water was measured in 1 ml of Cocktail T liquid scintillant (BDH Chemicals Ltd.) using a LKB/Wallac liquid scintillation counter, 1216 Rackbeta (LKB Produkter, Bromma, Sweden). All experimental points were determined in triplicate. The concentration of immunotoxin that inhibited [^{3}H]leucine uptake by 50% (the IC_{50} value) was calculated as described by Thorpe et al. (23).

Blood Clearance of Unconjugated and Saporin-conjugated Antibody. Male strain 2 guinea pigs were injected intravenously with 140 μg per 400 g body weight of antibody alone or antibody conjugated to 30 μg of saporin in PBS. At various intervals duplicate blood samples (50 μl) were taken in heparinized capillary tubes from the ear vein and diluted into 200 μl of supplemented DMEM containing 25 U/ml heparin (Weddel Pharmaceuticals Ltd., Wrexham, United Kingdom). After centrifugation at 500 g for 5 min, the supernatant was removed for assessment of antibody and immunotoxin content.

Antibody activity was measured in a standard ELISA as described by Stevenson et al. (31). Briefly 96-well microplates were coated with L₂C Id IgM at 100 ng/ml in sodium carbonate buffer, pH 9.6. After blocking with 1% BSA in PBS and washing, 100 μl aliquots of antibody standards (1–200 ng/ml) or diluted test samples were added, and bound mouse Ig detected by exposure to appropriately diluted horseradish peroxidase (HRP)-conjugated rabbit anti–mouse Ig (Nordic Laboratories Ltd., Maidenhead, United Kingdom).

The level of biologically active immunotoxin in the circulation of guinea pigs was assessed by measuring the ability of plasma samples to inhibit the incorporation of [^{3}H]-leucine into cultured L₂C cells as described above. The reduction in [^{3}H]leucine uptake induced by each plasma sample was compared with that induced by a range of immunotoxin standards to estimate the level of biologically active immunotoxin in the circulation.

Immunoglobulin Secretion by Cells in Culture. Fresh leukemic cells were suspended in supplemented DMEM at 2.5×10^6 cells/ml and incubated at 37°C in 80-cm² tissue culture flasks (Gibco) in a humidified atmosphere containing 5% CO_2 in air. Samples of the supernatant were taken at intervals, cooled on ice, and the cells sedimented by centrifugation at 200 g for 5 min. The concentration of guinea pig Ig in the supernatants was estimated by ELISA using the method described by Stevenson et al. (31). The IgM was captured from supernatant samples by adsorption binding to sheep anti-Fdμ (32) that had been coated onto microplates at 20 μg/ml and then detected with HRP-labelled sheep anti–μ chain antibody (31) at 4 μg/ml. Free guinea pig λ chain and IgM, both bearing the

L_2C Id, were quantified in similar assays, but using monoclonal anti–free λ Id and anti-Id-3, respectively, coated to the plate at 5 μg/ml, and then detecting the bound guinea pig Ig with an appropriately diluted HRP-labelled goat anti–guinea pig globulin antiserum (Nordic Laboratories Ltd., Maidenhead, United Kingdom). Standard solutions of guinea pig normal IgM (32), purified idiotypic IgM (25), and L_2C urinary λ chain (34) were included in all assays, over a concentration range of 1–200 ng/ml.

Immunotherapy Studies. Groups of age-matched strain 2 guinea pigs (400–450 g) were inoculated intraperitoneally with 10^5 L_2C cells (day 0). The doubling time of our L_2C tumor line is ~19 h, and without treatment animals die 13–15 d later. At this time the white blood count is ~250,000 cells/μl and the animals have extensive tumor deposits in the liver, lymph nodes, bone marrow, and particularly in the spleen. Treatment was given as a single intraperitoneal or subcutaneous injection on day 1 (unless stated otherwise), and consisted of specific immunotoxin, or of control reagents. Survival was monitored daily and expressed as a mean survival (days) for each group of animals. For the purposes of this calculation, the survival time of animals that did not develop tumor within a 100 d was taken as the last day on which a death occurred within that group.

Results

Toxicity and Specificity of Immunotoxin for L_2C Cells in Culture. Saporin has been conjugated to three IgG1 mAb for the present work. Two of these, anti-Id-1 and -3, are specific for the L_2C IgM Id and have previously been shown to have association constants at 37°C of 1.1 $\times$ 10^9 and 2.3 $\times$ 10^8/M, respectively (27). The third antibody was a control IgG1 of irrelevant specificity and showed no binding to L_2C cells. The toxicity of saporin-containing immunotoxins was assessed according to their ability to inhibit the incorporation of [^{3}H]leucine into protein by L_2C cells in vitro.

Fig. 1 *A* shows that both the anti-Id containing immunotoxins were highly toxic, halving the uptake of [^{3}H]leucine (IC_{50}) at a concentration of 4–8 $\times$ 10^{-11} M. In contrast, the IC_{50} for the control IgG–saporin conjugate and for free saporin were 2.8 $\times$ 10^{-8} M and 4.7 $\times$ 10^{-7} M (extrapolated value) respectively, indicating their comparative lack of toxicity. Saporin coupled to the control IgG was always 5–10-fold more toxic than free saporin, as shown in this experiment.

The high level of specificity indicated by toxicity results was confirmed using an L_2C variant, LIO1, which expressed normal levels of surface IgMλ with a mutant Id (described fully later) still recognized by anti-Id-3 but not by anti-Id-1. Fig. 1 *B* shows that this change in reactivity is accompanied by a corresponding change in sensitivity to the immunotoxins. The anti-Id-3 immunotoxin remained fully toxic for the variant cells (IC_{50} 2.2 $\times$ 10^{-10} M), while the nonreactive anti-Id-1–saporin conjugate had the same low toxicity as the control IgG immunotoxin.

Throughout this work, treatment of L_2C cells with anti-Id alone had no significant effect on [^{3}H]leucine uptake. Furthermore a mixture of antibody and free saporin, in proportions that matched those in the conjugate, was no more toxic than free saporin (Fig. 1 *A*).

Blood Clearance of Unconjugated and Saporin-conjugated Antibody. A small dose (140 μg) of unconjugated anti-Id-3 antibody (assayed as anti-Id antibody) disappeared from the plasma of guinea pigs with a half-life of 40 h (Fig. 2). In contrast, a similar quantity of the same anti-Id coupled to saporin (assayed as biologically active immunotoxin) was cleared more quickly, with the curve appearing biphasic. During the first phase the level of functional conjugate in the plasma fell away
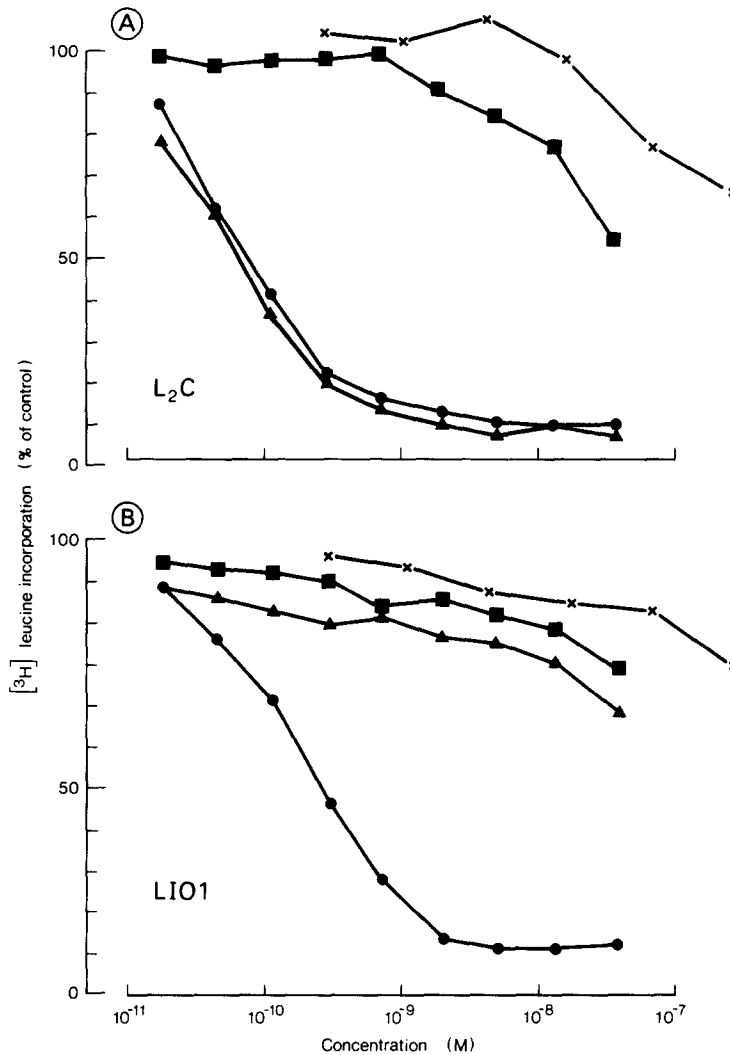
FIGURE 1. Uptake of [^{3}H]leucine by wild-type L$_2$C (A) and an idiotope-loss variant of L$_2$C, LIO1 (B), in culture. Cells (5 × 10^6 cells/ml) in supplemented DMEM were exposed to free saporin (X), control IgG–saporin (■), anti-Id-1–saporin (▲), or anti-Id-3–saporin (●) at the concentrations of saporin shown for 3 h at 37°C before pulsing with [^{3}H]leucine (6 h), and then harvesting the incorporated radioactivity. Points represent geometric means of triplicate determinations. Nonconjugated antibodies were without significant effect of [^{3}H]leucine uptake.

rapidly (half-life, 1–3 h), until at 6 h only ~10% of the initial conjugate remained. During the second phase this remaining 10% was cleared, but with a longer half-life of between 15 and 17 h (Fig. 2). By 50 h, circulating immunotoxin was generally present at <1% of its initial level. Unconjugated saporin can be expected to be cleared with a half-life of approximately 10 min (Stirpe, Derenzini, and Berbieri, manuscript in preparation).

Therapeutic Performance of Anti-Id Antibody and Anti-Id–Saporin Conjugates. Both the anti-Id antibodies (anti-Id-1 and -3) when given as unconjugated
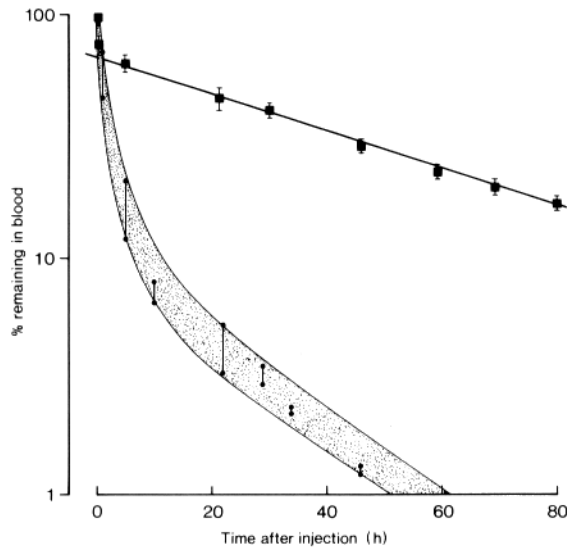
FIGURE 2. Blood survival of nonconjugated anti-Id-3 (■) and anti-Id-3–saporin conjugate (●) in strain 2 guinea pigs. After intravenous infusion of 140 µg antibody, plasma samples were taken at the times indicated and assessed for anti-Id antibody (■; determined by ELISA) or for the levels of functional immunotoxin (■; determined by the ability of plasma samples to inhibit [³H]-leucine uptake by L₂C cells). The bar at each point represents values determined for two animals (samples taken in duplicate). The levels of functional immunotoxin in the circulation proved difficult to determine and are subject to considerable error, but the actual rate of clearance probably lies within the range indicated (shaded area).
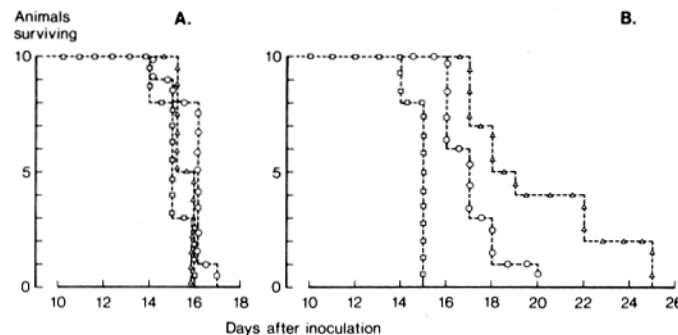

FIGURE 3. Immunotherapy with unmodified anti-Id mAb. Groups of 10 guinea pigs were inoculated intraperitoneally with 10⁵ L₂C cells (day 0). After 24 h they were injected subcutaneously with 150 µg (A) or 1 mg (B) of control IgG (□), anti-Id-1 (△), or anti-Id-3 (○). Survival was recorded daily.

preparations were able to prolong the lives of L₂C leukemic guinea pigs. Fig. 3B shows that a single subcutaneous injection of anti-Id-1 (1 mg) given 24 h after an intraperitoneal inoculum of 10⁵ L₂C cells increased the mean survival of animals from 14.8 d to 20 d. Anti-Id-3, although protective at 1 mg/animal in leukemic guinea pigs, was consistently less effective than anti-Id-1. Treatment with smaller quantities of either antibody yielded progressively less protection down to a level of ~150 µg/animal, at which point no increase in survival was seen (Fig. 3A).

Conjugation with saporin greatly improved the antitumor activity of the two anti-Id antibodies. In parallel experiments to those described above, the subcutaneous administration of only 140 µg of either antibody conjugated to ~30 µg of saporin extended the mean survival time of animals from 15.3 d in control animals to 30.5 and 30.8 d (Fig. 4A). In these experiments, unlike those using the unconjugated anti-Id antibodies (Fig. 3B), the two anti-Id conjugates performed equally. Other control groups evaluated in this investigation but not
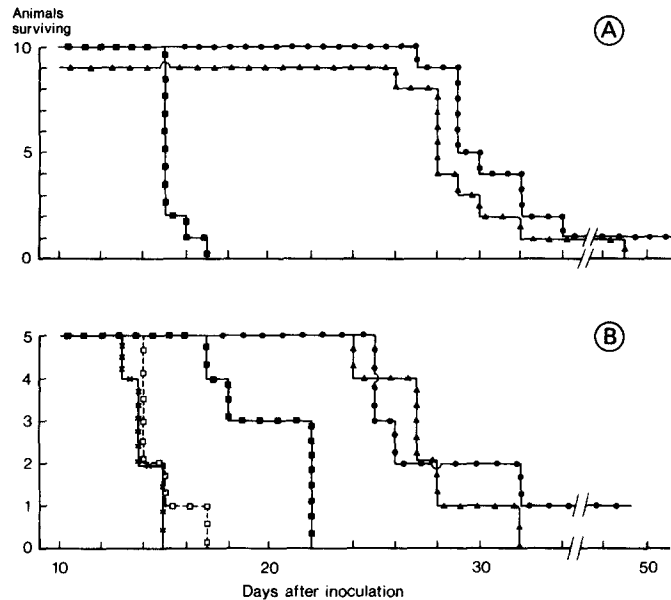
FIGURE 4. Immunotherapy with immunotoxins. Groups of guinea pigs were inoculated intraperitoneally with 10^5 L₂C cells (day 0). After 24 h they were injected subcutaneously (*A*) or intraperitoneally (*B*) with 170 μg of control IgG–saporin conjugate (■), anti-Id-1–saporin conjugate (▲), or anti-Id-3–saporin conjugate (●). Two other groups were treated with 1 mg of control IgG (□) or with 30 μg of free saporin (×). Survival was recorded daily.

shown in Fig. 4*A* included animals treated with free saporin (30 μg/animal), anti-Id alone (150 μg/animal), or a mixture of free saporin and unconjugated anti-Id given in the above proportions. All treatments were without significant protection against the leukemia, with mean survivals between 14 and 17 d.

Throughout these immunotherapy trials the L₂C cells and the immunotoxins were introduced at separate sites, using intraperitoneal and subcutaneous routes, respectively. When the immunotoxins and the tumor were both injected into the peritoneum, some protection was conferred by the control immunotoxin. Fig. 4*B* shows that in animals given the specific anti-Id immunotoxins the increase in mean survival remained unaltered at almost 14 d, while in those receiving conjugates containing control IgG it was increased from the usual 14 d to just over 20 d. Other control groups including those receiving free saporin or unconjugated control IgG via the peritoneum were not protected in this nonspecific manner (Fig. 4*B*).

Emergence of Ig⁻ L₂C During Therapy. Immunoglobulin expression on L₂C cells that emerged in guinea pigs after therapy was examined by cytofluorimetry using the FACS III. Leukemic cells recovered from animals that had been treated with control IgG alone, anti-Id antibody (1 mg/animal), or control IgG conjugated to saporin (Fig. 4) all had the normal Ig profile (Fig. 5*A*): strong reactivity by indirect immunofluorescence with anti-λ chain, anti-μ chain, and both anti-Id antibodies. In contrast, 16 of the 19 animals treated with the anti-Id–saporin conjugates developed tumors that were completely negative for both surface
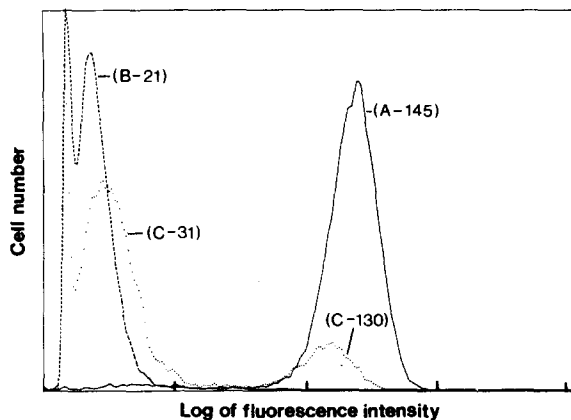
Log of fluorescence intensity

FIGURE 5. Expression of surface IgMλ on leukemic cells emerging after immunotherapy treatments described in Figs. 3 and 4. Cells (2 × 10⁷ cells/ml) were exposed to anti-μ mAb (5 μg/ml) and, after washing, bound mouse antibody was detected by fluorescent rabbit anti–mouse Ig. Three profiles of Ig expression were detected by cytofluorimetry: A, cells recovered from animals treated with control IgG (Fig. 3), unmodified anti-Id (Fig. 3), free saporin, or the control IgG-saporin conjugate (Fig. 4A); B, cells recovered from 16 of the 19 animals treated with the two anti-Id–saporin conjugates (Fig. 4A); and C, mixed cell populations recovered from 2 of the 19 animals treated with the anti-Id–saporin conjugate. Values in parentheses alongside each cell population represent the mean fluorescence intensity (arbitrary scale: 0–250) of that population. Similar training profiles were obtained using anti-λ and anti-Id mAbs (data not shown).

IgMλ and Id (Fig. 5B). Furthermore, this negativity was maintained during short-term culture in vitro (24 h) and for six serial passages in vivo, demonstrating that it cannot be attributed to modulation. The remaining two animals in which tumor developed from this group showed a mixture of surface Ig–negative and –positive cells. The positively staining cells also reacted with anti-Id antibodies. A typical surface Ig profile from one of these animals is shown in Fig. 5C. At this point the Ig⁻ variants were designated RN (receptor negative).

The RN cells were next investigated with respect to their production and release of Ig during short term culture in vitro. Serial supernatants were taken and assayed by ELISA for guinea pig IgM, free λ chain carrying the L₂C light chain Id, or Ig with the intact L₂C Id composed of the combined heavy and light chain. Fig. 6 shows that, unlike the wild-type L₂C, RN cells release no μ chain and consequently no intact idiotype into the supernatant. They do, however, secrete normal or even elevated levels of idiotypically intact λ chains.

The loss of Ig μ chain from RN cells was confirmed by showing that acetone-fixed cytospin preparations of RN cells were also negative when stained by the immunoperoxidase technique for intracellular μ chain. In addition, RN cells, consistent with their lack of surface Ig, were extremely resistant to the toxic effects of anti-Id-containing immunotoxins in vitro. Treatment of the cells with the immunotoxins at concentrations approaching 10^{-6} M reduced their [³H]-leucine incorporation by only 20–30%.

Emergence of Ig Variants after Immunotoxin Treatment In Vitro. To investigate further the heterogeneity of the L₂C leukemia with respect to Ig expression, fresh cells (2 × 10⁷ cells/ml) in supplemented DMEM were treated in vitro (2 h at 37°C) with the various immunotoxins (2 μg/ml) and were then given to untreated guinea pigs as an intraperitoneal inoculum of 4 × 10⁷ cells. The treated L₂C cells remained >95% viable by trypan blue exclusion at the time of inoculation and were injected without washing; each guinea pig thus receiving ~4 μg of immunotoxin along with the cells. Table I shows that animal survival was
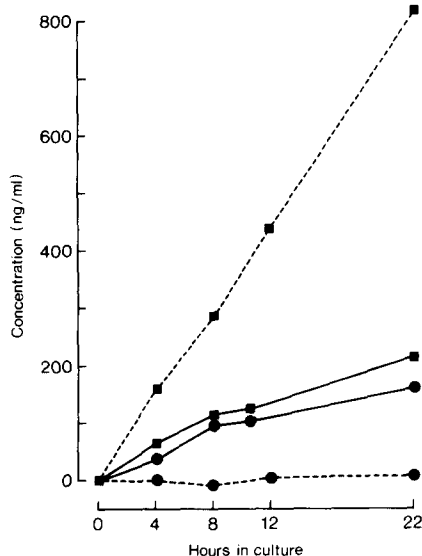
FIGURE 6. Immunoglobulin secretion by the wild-type L₂C cells (*solid lines*) and the surface Ig–negative variant, RN (*broken lines*). Cells were cultured (37°C) at 2.5 × 10⁶ cells/ml in supplemented DMEM, while aliquots of supernatant were taken at the times shown. Levels of free idiotypic λ chain (■) and μ chain (●) were measured by ELISA. Anti-Id-3 antibody was used in an additional ELISA to measure the intact Id formed by the combined μ and λ chains. However, for clarity, the results have not been included because they simply confirmed the release of intact IgMλ from wild-type L₂C (25) and showed that RNO1 cells failed to secrete detectable levels of intact Id.

TABLE I

Survival of Guinea Pigs Receiving In Vitro–treated L₂C Cells

Treatment	Mean survival (range)
	d
None	13.7 (13–15)
Anti-Id-1–saporin*	25 (22–27)
Anti-Id-3–saporin*	23.8 (23–25; one long-term survivor)

Matched groups of guinea pigs were given 4 × 10⁷ treated L₂C cells as an intraperitoneal inoculum on day 0, and survival times were recorded daily. For all groups, n = 5 animals.

* Cells at 2 × 10⁷ cells/ml in supplemented medium were treated in vitro for 2 h at 37°C with the anti-Id–saporin conjugates at 2 μg/ml.

extended by ∼10 d as a result of in vitro treatment, with one long-term survivor in which tumor failed to develop.

The predominant cell type (70–90%) to emerge in all these animals was completely negative for surface IgMλ and Id (Fig. 7), resembling the RN variant we had seen in our previous immunotherapy experiments with anti-Id-containing immunotoxins (Fig. 4). In addition to the RN cells, eight of the nine animals in which tumor developed also carried a minor population (4–26%) of cells expressing surface IgMλ (IgMλ⁺). However, in contrast to the IgMλ⁺ cells emerging in the previous experiments, these cells did not react with the therapeutic anti-Id antibody. Fig. 7 shows two such examples, which are typical of the cells recovered from the eight guinea pigs. In the first (Fig. 7A), ∼18% of the emerging L₂C express surface IgMλ, but the majority of these cells were unable to bind either of the anti-Id antibodies. Such cells probably represent a variant population of L₂C with a IgMλ⁺/Id-3⁻/Id-1⁻ phenotype; i.e., an idiotope-loss variant. A small number of cells (<2%) could be detected within this tumor sample that bound both anti-Id antibodies. These are probably residual phenotypically wild-type L₂C that survived the in vitro treatment with saporin conjugate.
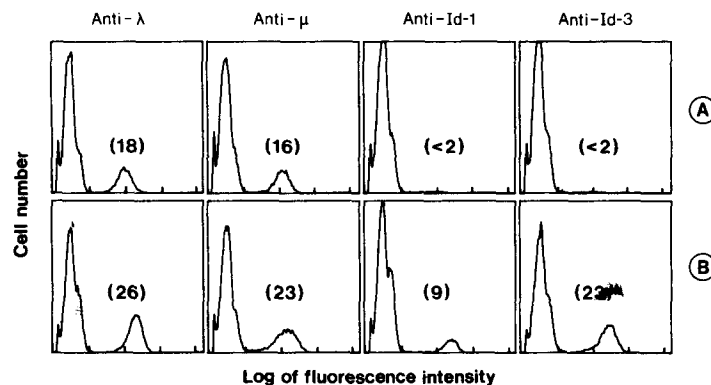
FIGURE 7. Expression of surface Ig on cells emerging in animals given in vitro–treated L_2C. Fresh L_2C cells (2×10^7 cells/ml) were treated with immunotoxins (2 μg/ml) for 2 h at 37°C as shown in Table I, and then inoculated into untreated strain 2 guinea pigs (4×10^7 cells/animal). Emerging leukemic cells were stained by indirect immunofluorescence using anti-λ, anti-μ, anti-Id-1, and anti-Id-3 followed by fluorescent rabbit anti-mouse Ig, before analyzing on the FACS III. Two typical staining profiles are represented from the nine animals that developed tumors (Table I): A, cells treated with anti-Id-3–saporin conjugate; B, cells treated with anti-Id-1–saporin conjugate. Values in parentheses indicate the percentage of cells staining with each antibody.

The second example in Fig. 7 (B) again shows a predominantly $IgM\lambda^-$ tumor (75%) that contained a minority population of L_2C that expressed surface $IgM\lambda$ (23–25%). However, in this example a similar proportion of cells (23%) also reacted with the anti-Id-3 antibody, while only 9% were able to bind anti-Id-1. These results point to the existence of at least two types of $IgM\lambda^+$ L_2C within this tumor: first a population of the cells (9%) that were recognized by both anti-Id antibodies, and which probably represent wild-type L_2C cells not destroyed by immunotoxin treatment; the second is a population representing ~14% of the tumor, which expressed the determinant seen by anti-Id-3 but not that recognized by anti-Id-1 ($Id-3^+/Id-1^-$). These $Id-3^+/Id-1^-$ cells were recovered in four of the five animals that received tumor treated with the immunotoxins containing anti-Id-1 (Table I). The reciprocal phenotype, $Id-3^-/Id-1^+$, was not observed after treatment with the anti-Id-3–saporin conjugate. This latter treatment yielded only the $IgM^+/Id-3^-/Id-1^-$ variant shown in the first example.

Isolation of Idiotope-loss Variants. The tumors that emerged containing Ig^- and idiotope-loss variants were each passaged without further treatment for one generation in guinea pigs. They were then labelled by indirect immunofluorescence using anti-λ and anti-μ antibodies, together with a panel of 14 different anti-L_2C antibodies. All tumors retained their altered Ig phenotypes, but the proportion of $IgM\lambda^+$ cells was increased to almost double, suggesting that these cells have greater vigor. Fig. 8 shows a typical example of the staining profile obtained with these passaged tumors. These particular cells correspond to those shown in Fig. 7A. The bulk of the tumor remained Ig^- (RN), but the proportion of $IgM\lambda^+$ cells increased from ~17% on first emerging (Fig. 7A), to ~30% in the second guinea pig. Although 10 of the 14 anti-Id antibodies, including anti-Id-1 and -3, showed no significant labelling of these cells, the remaining 4 stained a similar proportion to the anti-λ and anti-μ antibodies. Note that those cells that
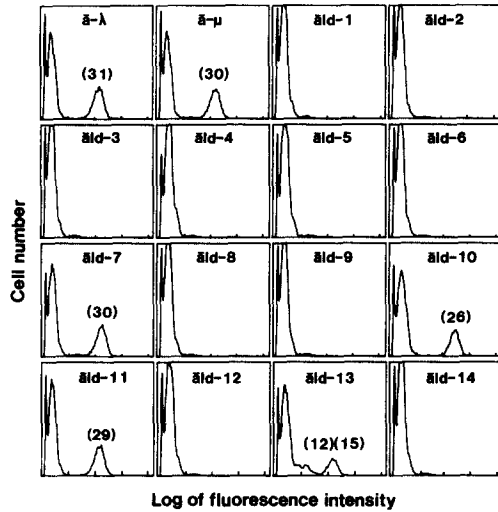
FIGURE 8. Identification of idiotype-loss variants emerging in animals given in vitro–treated L₂C. The leukemic cells shown in Fig. 7A were passaged in a single untreated guinea pig before being analyzed by indirect immunofluorescence using monoclonal anti-λ and anti-μ, together with a panel of 14 anti–L₂C Id mAbs (anti-Id-1–14). Values in parenthesis indicate the percentage of cells staining with individual mAbs.

Antibody	Variants						
	L₂C	LI01	LI02	LI03	LI04	LI05	LI06
Anti-λ	139	140	140	141	142	140	139
Anti-μ	144	144	140	139	139	140	144
Anti-Id-1	150	41	47	35	34	37	38
Anti-Id-2	150	140	48	36	31	33	36
Anti-Id-3	140	148	150	118	43	36	34
Anti-Id-4	147	149	151	37	31	33	90
Anti-Id-5	138	147	144	39	34	36	32
Anti-Id-6	149	151	148	134	33	36	39
Anti-Id-7	148	149	150	153	143	142	36
Anti-Id-8	144	38	46	144	31	34	36
Anti-xId-9	129	144	139	138	34	36	37
Anti-Id-10	131	152	155	134	142	142	158
Anti-Id-11	124	40	46	147	119	136	38
Anti-Id-12	153	152	75	62	33	34	36
Anti-Id-13	142	144	148	137	123	84	138
Anti-Id-14	144	143	47	37	31	34	38

FIGURE 9. Variants (LIO1-6) isolated from tumors emerging in guinea pigs given immunotoxin-treated L₂C (Table I) were characterized with respect to their reactivity with anti-λ and anti-μ mAb together with a panel of 14 anti–L₂C-Id antibodies. Indirect immunofluorescence staining was measured on the FACS III. Numbers are mean fluorescence intensity (arbitrary scale: 0–250) for each antibody–variant reaction. The shaded bars represent the intact L₂C IgM Id, loss of recognition by a particular antibody (mean fluorescence intensity <50) is indicated by an unshaded box in the bar.

reacted with anti-Id-13 could be subdivided into at least two populations: those which stained strongly and those which stained weakly.

Using the reactive anti-Id antibodies for each of the passaged tumors, the idiotope-loss variants were stained and then sorted on the FACS III. The isolated cells were expanded in untreated guinea pigs before being resorted and passaged

in guinea pigs. In this way, six distinct idiotope-loss variants (LIO1–6) were eventually isolated to homogeneity, each showing a different reaction profile with the panel of antibodies (Fig. 9). At the extremes of these reaction profiles were LIO1, which retained reactivity with 11 of the 14 antibodies, and LIO6, which bound only 3. While certain variants appear to retain reactivity with antibodies, the intensity of the staining was sometimes well below average. A good example of this was the labelling of LIO5 cells with anti-Id-13. This antibody binds to LIO5 cells, but with a mean fluorescence intensity approaching half its usual level. These particular cells can also be seen before they were sorted as the weakly staining population āId-13 of Fig. 8.

Despite careful analysis, we have not isolated cells that express Ig with a completely novel idiotype, i.e., which fail to react with any of the anti-Id antibodies in the panel.

Discussion

The covalent linkage of the RIP, saporin, to anti-Id mAb has been shown to generate highly potent and specific reagents. Both the anti-Id antibodies (anti-Id-1 and anti-Id-3) have produced immunotoxins that were toxic for L_2C cells in vitro at concentrations below 10^{-10} M ($IC_{50} = 4$–8×10^{-11}), while those containing a control IgG showed little toxicity unless added at concentrations above 10^{-7} M. The high degree of specificity was confirmed using an Id variant of the L_2C tumor that was recognized by anti-Id-3 but not by anti-Id-1.

It is known that the binding affinities of the two therapeutic anti-Id antibodies we have used differ by a factor of 10 (see Results); however this difference does not seem to have influenced the specific cytotoxicity of the immunotoxins in vitro. Other workers (22) have found that cytotoxic potency increases as the affinity of the antibody component increases. It is possible that the lower-affinity immunotoxin used in the present study recognized an epitope that was particularly favorable for immunotoxin activity, for example, one that encouraged the immunotoxin to enter the cell via endocytic vesicles (27), or one that positioned its RIP component in close proximity to the membrane it must traverse.

The short metabolic survival of our immunotoxins in guinea pigs compared with that of the nonconjugated antibody was not unexpected. Other workers have found that conjugation of proteins, including RIPs, to antibodies considerably reduces their survival in the circulation (35–37). In the present studies we have shown that ~5% of the initial levels of functional anti-Id–saporin conjugate remained in the plasma at 24 h, and that cytotoxic levels ($>10^{-10}$ M) were detectable for ~2 d after the infusion of 350 μg/kg body wt. Similar results have been reported by Letvin et al. (36) after administration of monoclonal anti-T11–saporin and –gelonin conjugates to rhesus monkeys. They found that in the initial decline phase, the circulating levels of intact immunotoxins dropped to ~10% of their original levels. Thereafter cytotoxic immunotoxin remained detectable in the plasma for up to 3 d.

At least two factors are known to limit the survival of immunotoxins in animals and probably also in man: first, the rapid removal by the liver, the organ in which most toxicity has been reported; and second, the breakdown of the disulfide linkage between the antibody and the RIP. The accumulation in the

liver has been a particular problem for those RIPs such as ricin A chain, which are naturally glycosylated. Specific hepatic receptors for carbohydrate residues (such as mannose) can remove these compounds very effectively, giving them very little chance to circulate in the plasma (37) unless they are first deglycosylated (38). Saporin does not suffer from this problem, as it is free of carbohydrate (33), but nevertheless when linked to IgG or F(ab')$_2$ it still accumulates in the liver, where considerable toxicity may result (Stirpe, Derenzini, and Berbieri, manuscript in preparation). The lack of stability in the disulfide linkage between the antibody and RIP is also a problem with immunotoxins prepared using commercially available crosslinking agents, such as SPDP and 2-iminothiolane (36, 38). Recently this problem has been tackled by the synthesis of new disulfide coupling agents that form immunotoxins relatively stable in vivo (Wallace and Thorpe, manuscript in preparation).

Therapeutically, the anti-Id immunotoxins used in the present study have proved potent agents. A single dose of immunotoxin extended the lives of leukemic animals by ~15 d. Unmodified anti-Id antibodies have never approached this level of protection in the L$_2$C leukemia (39). We have shown in the present work that the maximum increase in mean survival seen with mouse mAbs was ~5 d (Fig. 3B). The proportion of the L$_2$C inoculum destroyed during such treatment cannot be measured accurately. It might be assumed that, with a doubling time of ~19 h, a 50% reduction of the tumor load would protect animals carrying the L$_2$C leukemia for this period. Thus, any treatment that provides an extra 15 d survival will probably need to destroy all but a minute fraction of the initial tumor inoculum. Indeed, if we omit complicating factors from our calculations, such as changes in the doubling time of the tumor during its development, then it can be estimated that only one cell need escape the immunotoxin treatment. This estimate accords with the results of Thorpe et al. (23), who showed that a single injection of an anti-Thy-1.1–saporin immunotoxin into mice bearing a Thy-1.1-expressing lymphoma extended the survival times of the animals by the extent expected if 99.999% of the tumor cells had been destroyed. Similarly, in an earlier study, Vitetta and colleagues (40) showed that mice with advanced B cell lymphoma (BCl$_1$) could be rendered free of apparent disease by splenectomy, total lymphoid irradiation, and treatment with an immunotoxin composed of anti-IgD antibody and ricin A chain. The surviving mice, however, harbored a number of dormant tumor cells that could induce lymphoma when transplanted into naive recipients (41). Several other groups have also shown impressive antitumor effects with immunotoxins, but in almost all cases animals have not been entirely cleared of disease (19).

Ig phenotypes of the cells emerging after therapy with immunotoxins are summarized in Table II. Tumors emerging in leukemic animals that had been treated with anti-Id–saporin conjugate were predominantly (16 of 19 animals) IgMλ$^-$ (Table II, protocol A). This phenotype was not due to chronic modulation of cells in the treated animals, as it was maintained during short-term culture in vitro and stable through six serial passages in untreated animals. Further analysis of the Ig production by these cells, which we have termed RN, revealed them to be μ heavy chain–loss mutants of the L$_2$C line, which neither expressed nor exported intact IgM. Their Ig loss did not extend to the λ light chain, which was

TABLE II

Summary of Tumor Variants Arising after Anti-Id Immunotoxin Treatment

Cell population	Protocol	
	A	B
Cells inoculated into each guinea pig	10^5 (untreated)	4×10^7 (cells pretreated with anti-Id–saporin)
Treatment of tumor-bearing animals	Anti-Id–saporin (170 μg/animal)	None
Emerging tumor cells	Majority surface Ig–negative	Majority surface Ig–negative
	Small minority surface Ig–positive (Id-intact)	Minority surface Ig–positive (Id-mutated)
		Small minority surface Ig–positive (Id-intact)

In protocol A, guinea pigs that received L_2C cells (intraperitoneally) 24 h previously were treated with a single subcutaneous injection of anti-Id immunotoxin (170 μg/animal), while in protocol B, fresh L_2C were exposed to the anti-Id immunotoxin (2 μg/ml) for 2 h in vitro (37°C) before being transferred into guinea pigs.

secreted normally as free molecules with a light chain idiotype characteristic of the parent tumor.

A number of animal studies have demonstrated that immunological pressure on a tumor can result in the outgrowth of stable variants that lack the target antigen. This event has been described in at least two situations: in tumor-bearing animals that have been previously immunized with the tumor (7) or with an appropriate antigen derived from the tumor (6); and after passive serotherapy with specific antitumor antibody (4, 5). In the L_2C model, despite some highly significant tumor protection, neither of these approaches has yielded stable L_2C variants. Stevenson and Gordon (42) showed that the leukemias that emerged in guinea pigs that had been preimmunized with L_2C derived idiotypic IgM were often negative for surface IgM. However this phenotype resulted not from a tumor variant but from antigenic modulation, which was reversed on removal of the immunological pressure either by culturing cells in vitro or passage in naive animals. Similarly, multiple infusions of an anti-Id mAb given to animals bearing this tumor also yielded cells that had modulated their surface IgM, a condition they maintained only while excess antibody persisted in the animals to perturb the turnover of newly synthesized surface IgM molecules (43).

The emergence of RN as a stable IgM⁻ variant(s) of the L_2C implies that the current immunotoxin therapy has imposed a greater selective pressure on this tumor than our previous regimens. This increased potency probably stems in part from the fact that antigenic modulation does not offer an effective means of escape to tumor cells, and may actually aid their destruction by channelling the saporin into the endosomes (44, 45). The frequency at which RN cells exist in the L_2C population is unknown. Clearly it is well above 10^{-5}, because all but 1 of the 19 animals treated developed tumors that were totally (16 animals) or partially (2 animals) composed of RN cells. Further studies are underway to

establish this frequency and also to show whether RN cells can arise continuously in the L₂C tumor.

The second type of Ig variant to be selected by immunotoxin treatment expressed normal levels of surface IgMλ, but failed to react with the monoclonal anti-Id used in the therapeutic conjugate; i.e., they were idiotope-loss variants. Such cells must be at a very low frequency in the wild-type L₂C population, because they only became apparent after the in vivo passage of a large tumor inoculum (4 × 10⁷ cells) that had been preexposed to an immunotoxin in vitro (Table II, protocol B). Careful analysis and sorting by cytofluorimetry using a panel of anti-Id mAbs allowed six distinct and stable idiotope-loss variants (LIO1–6) to be isolated. Each reacted with anti-λ and anti-μ antibodies together with various combinations of anti-Id in the panel, but failed to bind the particular anti-Id (anti-Id-1 and/or -3) from which they had escaped during treatment. A wide spectrum of reactivity was observed, from LIO1 which bound 11 of the 14 anti-Id, to RNO6, which reacted with only 3.

These changes in idiotype expression appear analogous to those described by Meeker et al. (8) in two cases of B cell lymphoma receiving treatment with unmodified anti-Id antibody. In both situations the emerging tumors have failed to recognize the antibody that was used in treatment, despite the continued expression of surface Ig and the apparent monoclonal origin of these tumors. The Stanford group have pointed to extensive point mutations in the Ig V region of one of these tumors (9) and also in many other B cell neoplasms (Levy, R., personal communication) as an explanation for their loss of recognition by anti-Id antibody. A similar explanation for our current observation in the L₂C tumor would also implicate several changes in the V region sequence in order that at least six and probably more new idiotypes could be generated. However, alternative mechanisms may also exist in which more extensive changes in the Ig Id could occur. Studies in hybridoma cells (16), and recently with the chicken λ light chain V gene (46) have shown that whole segments of the V region DNA in a rearranged V gene can be replaced by that of neighboring V regions to generate new sequences. We suspect that such transpositions would introduce quite major structural alterations into all or at least a major part of the Ig V region. Our present work indicates that many of the changes in idiotype are quite subtle: first because some idiotope-loss variants retain activity with almost all the anti-Id antibodies (LIO1 and 2), and second because occasionally anti-Id antibodies bind with a reduced intensity to individual variants compared with their activity against the original tumor, suggesting that the idiotope remains with very minor changes. The exact mode and extent of changes in the V region sequence will only emerge when the L₂C and its variants have been fully analyzed at the level of the DNA.

The current work underlines the potential problems facing the Ig Id as a target for the treatment of B cell malignancies. As the selective pressure from antibodies is increased, perhaps by avoiding antigenic modulation or by using immunotoxins that circumvent this primary escape route, the next major mechanism of tumor escape becomes variants that have lost or mutated their Ig. Most evidence to date (6, 7, 47, 48) together with the current observations suggest that heavy chain–loss mutants will prove most frequent and troublesome. They pose a particular problem because the loss of surface Ig means that a completely

new tumor-associated target antigen must be sought to attack these variants. The idiotope-loss variants are potentially less troublesome because, as we have shown in the current work, while the surface Ig remains on a tumor, it should be possible to generate anti-Id antibodies or perhaps groups of antibodies that cover the complete spectrum of expressed idiotype.

Summary

The potency and specificity of immunotoxins consisting of monoclonal anti-idiotype conjugated to the ribosome-inactivating protein, saporin, have been evaluated in the treatment of guinea pig L_2C B lymphocytic leukemia. The immunotoxins were therapeutically much more effective than their parent antibodies. Their specificity reflected that of their antiidiotype component. Although the leukemia emerged eventually in most animals treated with these conjugates, most of the cells showed altered Ig expression, which rendered them resistant to the therapy. Commonly, the emerging cells had lost μ heavy chain production, leaving them negative for intracellular, surface, and secreted IgM, but still positive for λ light chain production. In addition, a minor group of L_2C variants was identified in a protocol designed to detect mutants at very low frequency: here the cells were exposed in vitro to immunotoxin and, while still viable as judged by dye-exclusion, inoculated in large numbers into animals. In tumor that emerged under these circumstances, the majority of cells were again immunoglobulin-negative; however a minority exhibited IgM with an altered idiotype (Idiotope-loss variants), rendering them unreactive with immunotoxin. Immunotherapy with unmodified anti-Id antibody alone does not reveal these variants, and we suggest it is the increased selective force exerted by the highly potent immunotoxins that allow these minor nonreactive populations to emerge.

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