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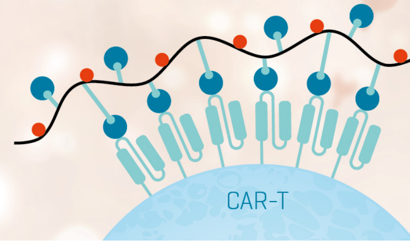

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A CHIMERIC IL-2/Ig MOLECULE POSSESSES THE FUNCTIONAL ACTIVITY OF BOTH PROTEINS

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An expression vector (pIL-2/IgG1) was constructed with the coding sequence of human IL-2 inserted upstream of the four exons (CH1, hinge, CH2, and CH3) that encode the human IgG1 H chain constant region. Introduction of this vector into a nonsecreting murine myeloma cell line resulted in the production of a chimeric molecule (IL-2/IgG1) consisting of IL-2 attached to the three Ig constant region domains. This molecule was secreted by the transfectant as a homodimer. Functional characterization revealed that the IL-2/IgG1 chimeric molecule exhibited the binding and proliferation-mediating activities of IL-2. On a per molecule basis, IL-2/IgG1 was indistinguishable from human rIL-2 in the ability to induce the proliferation of an IL-2-dependent T cell line. This chimeric molecule also possesses Ig effector function, in that it can mediate the specific lysis of IL-2R-positive cells in the presence of complement. These results demonstrate that it is possible to maintain Ig effector function in molecules ("immunoligands") in which the binding specificity is conferred not by Ig variable regions, but rather, by a ligand of choice.

The Ig molecule has been an important target for manipulation by protein engineering with the intent of creating potentially valuable agents for the treatment of human disease. This effort has resulted in the production of modified antibody molecules that have enormous therapeutic potential (1). The majority of this "antibody engineering" has involved maintaining the original specificity of an Ig variable region while altering the remainder of the molecule. These alterations have included attaching an enzyme (2, 3) or a toxin (immunotoxin) (4, 5) to all or part of an Ig molecule. Also, murine mAb that possess therapeutic potential have been modified by replacing the constant region (chimeric) (6, 7) or constant and framework regions (hyperchimeric or humanized) (8-10) with human sequences. Antibodies modified in such a manner retain their original specificity, but are expected to exhibit little or no immunogenicity in human patients (11, 12).

More recently, CD4/Ig chimeric molecules (immunoadhesins) (13-15) have been constructed by replacing the variable region of an Ig molecule with all or part of the extracellular portion of CD4. The CD4 molecule is a member of the Ig superfamily (16) and thus folds in a manner that is compatible with the Ig constant region (13-15). These chimeric molecules retain the binding specificity of CD4 for gp120 of the HIV and exhibit some Ig effector function (13-15). To determine if it is possible to maintain Ig effector function in molecules where the variable region is replaced by a domain structurally disparate to those of the Ig superfamily, an IL-2/IgG1 chimeric protein was constructed and characterized. This molecule possesses the functional activities of both IL-2 and IgG, demonstrating that it is possible to maintain Ig effector function in molecules ("immunoligands") in which the binding specificity is conferred by a domain that is not a member of the Ig superfamily.

MATERIALS AND METHODS

Vector construction. The pIL-2/IgG1 plasmid was constructed by changing the Xba I site of the vector pVγ_1 (10) to a Sal I site and inserting a 503 base pair fragment containing an altered form of the human IL-2 gene. The IL-2 gene employed differed from the natural sequence of human IL-2 (17) in that it encoded for an alanine rather than a cysteine at position 125. The IL-2 gene had also been modified using polymerase chain reaction (18) at the 3′ end by the addition of a single codon for serine followed by a splice donor site to allow the 3′ terminus of IL-2 to be spliced to the 5′ end of CH1. The splice donor site and the next 20 bases of the intron were identical to that found at the end of the murine J_H2 segment (19). A 235 base pair intron separated the IL-2 and IgG1 genes. A Sal I site was also added to each end of the fragment to facilitate insertion into the vector.

Electroporation, selection, and transfectant screening. Sp2/0 cells were transfected and selected for hygromycin B resistance (10). Supernatants from resistant cells were screened for the presence of molecules expressing human IgG determinants by ELISA utilizing affinity purified goat anti-human (Tago, Burlingame, CA) as both the capture and developing reagents. A transfectant registering positive in the assay was subcloned three times, resulting in the cell line 87.20.12.

Biochemical characterization of IL-2/IgG1. 10^7 87.20.12 cells were incubated for 12 h in 10 ml of DMEM + 10% dialyzed FCS containing 1 mCi [^{35}S]methionine. Supernatant from labeled cells was concentrated four-fold prior to use; labeled cells were lysed in 0.5% NP-40. Immunoprecipitation was carried out by incubating supernatant or lysate with the indicated antibody-coated agarose for 1 h at 4°C with agitation. The preclearing experiment was carried out by incubating labeled supernatant with an excess of goat anti-human IgG agarose for 3 h prior to immunoprecipitation. Immunoprecipitates were analyzed on either a 7.5% or 10% polyacrylamide gel under reducing conditions (20) unless otherwise indicated.

IL-2/IgG1 purification. Culture medium from 87.20.12 cells was concentrated approximately ten- to twenty-fold using an Amicon 8400 concentrator (Amicon, Danvers, MA). The concentrated material was passed over a Protein A agarose column (BRL, Gaithersburg, MD), and column adherent material was eluted with 0.1 M glycine, 0.15 M NaCl, pH = 2.5. Eluted fractions were immediately neutralized by the addition of 1 M Tris, pH = 9.6.

Functional characterization of IL-2/IgG1. Detection of IL-2/IgG1 binding to IL-2R-positive cell lines was carried out by indirect immunofluorescence as described (21), and cells were analyzed on a FACScan (Becton-Dickinson, San Jose, CA). The anti-Tac (22) and

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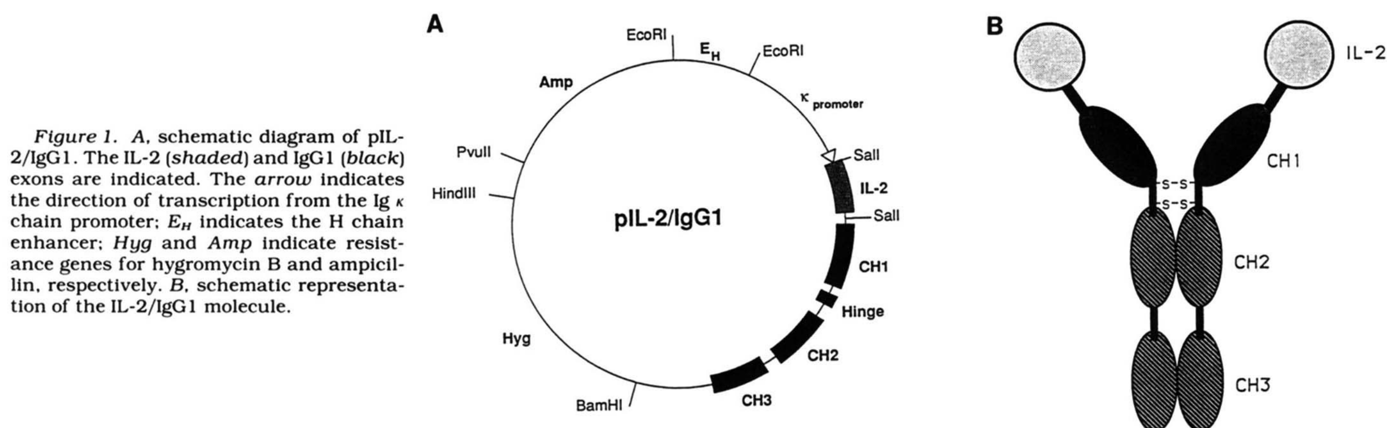

Figure 1. A, schematic diagram of pIL-2/IgG1. The IL-2 (*shaded*) and IgG1 (*black*) exons are indicated. The *arrow* indicates the direction of transcription from the Ig κ chain promoter; E_H indicates the H chain enhancer; *Hyg* and *Amp* indicate resistance genes for hygromycin B and ampicillin, respectively. *B*, schematic representation of the IL-2/IgG1 molecule.

PC 61 5.3 (23) mAb served as the positive control for detection of the p55 chain of the human and mouse IL-2R, respectively. IL-2 activity (stimulation of proliferation of CTLL cells (24)) was assayed by incubating 5×10^4 cells for 18 h with the indicated concentration of IL-2/IgG1 or human R IL-2 (Amgen, Thousand Oaks, CA) in a total volume of 200 μl. After an 18-h incubation, 1 μCi of [^{3}H]thymidine was added, and the cells were incubated for an additional 4 h. Samples were harvested and radioactivity was quantified by scintillation counting. All determinations were done in quadruplicate. Complement fixation was carried out by precoating 10^6 ^{51}Cr-labeled HuT-102B cells with the indicated concentration of anti-Tac or IL-2/IgG1 for 45 min at 4°C. The cells were washed twice and adjusted to 5×10^5/ml. One hundred μl of a 1:2 dilution of rabbit complement (Pel-Freeze, Rogers, AK) was added to 100 μl of cells, and the samples were incubated for 45 min at 37°C. NP-40 (1%) was substituted for the complement to determine the total radioactive release. Following incubation, one-half of the supernatant was removed to determine ^{51}Cr release. Specific lysis was calculated as described (25). All determinations were done in quadruplicate.

RESULTS

An expression vector (pIL-2/IgG1) was constructed that contains a slightly altered form of the coding sequence of human IL-2 upstream of four exons (CH1, hinge, CH2, CH3) encoding the human IgG1 H chain (Fig. 1*A*). Thus, the IL-2 gene was placed in the position normally occupied by the VDJ[2] Ig gene segment. The IL-2 gene differed from the natural human IL-2 sequence in that it encoded an alanine in place of a cysteine at position 125. Also, the 3′ end of the IL-2 gene was slightly modified by the addition of a codon for serine, followed by a splice donor site. The serine was added to make the carboxy terminus of IL-2 resemble the hydrophilic-large hydrophobic-hydrophilic-hydrophilic pattern of amino acids commonly observed at the carboxy end of human VDJ segments (26). A short intron separated the modified end of the IL-2 gene from the first of the IgG1 exons, which were in the genomic configuration (Fig. 1*A*).

The construct was introduced into the murine nonsecreting myeloma cell line Sp2/0 by electroporation. Supernatants from wells containing hygromycin B-resistant cells were screened for the presence of protein possessing human Ig determinants by ELISA. Approximately 5% of the transfectant supernatants registered positive in the ELISA, indicating the secretion of a molecule with human Ig determinants. Transfection into cell lines expressing an endogenous L chain gene did not increase the number of positive supernatants or the level of expression (data not shown). Subcloning of a positive Sp2/0 transfectant resulted in a cell line (87.20.12) that was more extensively characterized.

The 87.20.12 cell line was incubated with [^{35}S]methionine; the resulting supernatant was collected and a detergent lysate was prepared from the cells. Immunoprecipitation and PAGE analysis under reducing conditions revealed that both the supernatant and the cell lysate contained a molecule of approximately 55 kDa that was specifically precipitated by anti-human IgG-coated agarose (Fig. 2*a*, *lanes 2* and *6*). A molecule of identical molecular weight was immunoprecipitated by a rabbit anti-human IL-2 antisera (Fig. 2*a*, *lanes 3* and *7*) and by protein A-coated agarose (Fig. 2*a*, *lanes 4* and *8*). No molecules were specifically immunoprecipitated from a labeled lysate of untransfected Sp2/0 (data not shown). Preclearing the 87.20.12 supernatant with anti-human IgG-coated agarose removed the molecule reactive with the anti-IL-2 antiserum (Fig. 2*b*), demonstrating that the Ig and IL-2 determinants reside on the same molecule. Thus, the 87.20.12 transfectant secretes a chimeric IL-2/IgG1 molecule. To establish whether the molecule is secreted as a monomer or in a multimeric form, an immunoprecipitate from a labeled supernatant was electrophoresed under nonreducing and reducing conditions. The IL-2/IgG1 molecule analyzed under nonreducing conditions displayed approximately twice the molecular weight as that observed under reducing conditions (Fig. 2*c*), and thus is secreted by 87.20.12 as a homodimer, presumably disulfide linked between the Ig hinge regions.

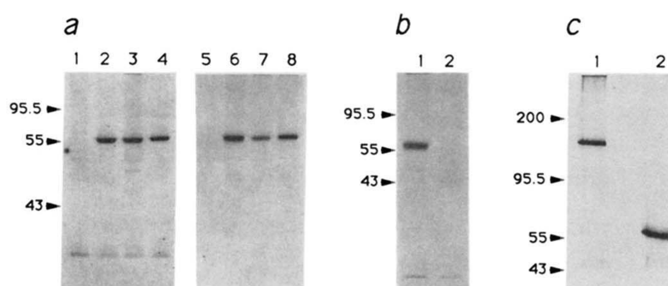

Figure 2. Biochemical analysis of the 87.20.12 transfectant. *a*, immunoprecipitation of the supernatant (*lanes 1–4*) and cell lysate (*lanes 5–8*) from ^{35}S-labeled 87.20.12 cells. Lanes 1 and 5 were incubated with rabbit IgG agarose, lanes 2 and 6, goat anti-human IgG agarose, lanes 3 and 7, rabbit anti-human IL-2 agarose, and lanes 4 and 8, protein A agarose. *b*, ^{35}S-labeled 87.20.12 lysate was cleared with rabbit IgG agarose (*lane 1*) or goat anti-human IgG agarose (*lane 2*), prior to immunoprecipitation with rabbit anti-IL-2 agarose (*lanes 1 and 2*). *c*, 87.20.12-labeled supernatant was incubated with goat anti-human IgG agarose and the immunoprecipitate was electrophoresed under nonreducing (*lane 1*) or reducing (*lane 2*) conditions. The positions of m.w. markers (in kDa) are indicated on the left of each panel.

[2] Abbreviations used in this paper: VDJ, variable-diversity-joining; ADCC, antibody-dependent cell cytotoxicity.

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A schematic representation of the IL-2/IgG1 chimeric molecule is presented in Figure 1*B*.

To determine whether the IL-2 portion of IL-2/IgG1 is in the native conformation, the ability of the chimeric molecule to bind to cells expressing the IL-2R was assayed by indirect immunofluorescence. Figure 3 compares the level of fluorescence observed using the chimeric molecule to that obtained using mAb against the p55 chain of the IL-2R. IL-2/IgG1 binding can be detected to the IL-2R expressing cell lines HuT-102B (human) and CTLL (murine), but not to the IL-2R-negative human T cell lines Jurkat and CEM. IL-2/IgG1 reactivity was also observed with populations of mitogen-activated murine and human T lymphocytes; preincubation of the cells with IL-2 significantly decreased the observed level of fluorescence (data not shown). These results indicate that the IL-2 portion of the chimeric molecule is in a configuration sufficient to allow binding to the IL-2R on the surface of cells. Furthermore, analogous to human IL-2, IL-2/IgG1 demonstrates the ability to bind both human and murine cell surface IL-2 receptors (20).

In order to determine whether the IL-2 portion of the chimeric molecule has complete functional activity, IL-2/IgG1 was purified from 87.20.12 supernatant by passage over a protein A column, and the purified material was analyzed for the ability to support the proliferation of the IL-2/IL-4-dependent murine cell line CTLL (24). The chimeric molecule could stimulate proliferation of this cell line, and comparison with the proliferation stimulated by human rIL-2 revealed that on a per molecule basis, chimeric IL-2/IgG1 has a specific activity that is indistinguishable, within the limits of this biological assay, from human rIL-2 (Fig. 4*A*). Furthermore, 100% of the proliferation-inducing capacity of the IL-2/IgG1 preparation can be removed by incubation with either a goat anti-human IgG agarose or a rabbit anti-human IL-2 agarose (Fig. 4*B*), indicating that all IL-2 activity is in the form of a chimeric molecule. Thus, the IL-2 moiety of the chimeric molecule is in a fully functional configuration, exhibiting both the binding and proliferation-mediating activities of IL-2.

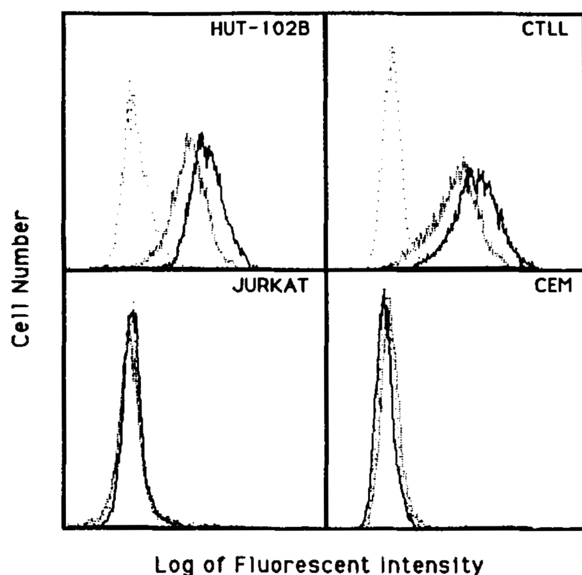

Figure 3. Cytofluorometric detection of the binding of IL-2/IgG1 to cell lines that express the IL-2R. Profiles of the indicated cells incubated with an irrelevant antibody (· · · ·); IL-2/IgG1 (········); humanized anti-Tac (10) (——) for HuT-102B, Jurkat and CEM, or PC 61 5.3 (23) (——) for CTLL, before incubation with the appropriate FITC-conjugated reagent (goat anti-human or goat anti-mouse IgG Fc). Histograms indicate the cell number versus the log of fluorescence intensity.

The functional status of the Ig portion of the IL-2/IgG1 molecule was also examined. The capacity for antibody effector function, such as complement fixation and the ability to mediate ADCC, resides in the Ig constant region (27). The human IgG1 isotype can mediate both of these effector functions (27). IL-2/IgG1 was compared with the murine anti-Tac mAb for the ability to mediate complement-dependent lysis of the HuT-102B cell line. IL-2/IgG1 clearly has the ability to specifically lyse HUT-102B cells in the presence of complement (Fig. 5*A*). The specificity of this serologic reaction is demonstrated by the inability of IL-2/IgG1 to mediate the lysis of Jurkat (IL-2R-negative) cells (Fig. 5*B*). IL-2/IgG1 was also examined for the ability to mediate ADCC. The murine anti-Tac mAb does not mediate ADCC, however, the chimeric and humanized versions of this antibody exhibit detectable levels of ADCC activity with the use of an activated effector cell population (25). IL-2/IgG1 and chimeric anti-Tac each exhibited a marginal (28% and 15%, respectively) enhancement of lysis of HuT-102B target cells in a 4-h ^{51}Cr release assay (data not shown). In conclusion, these results indicate that the chimeric IL-2/IgG1 molecule possesses the functional activities of both the IgG and IL-2 moieties.

DISCUSSION

The chimeric gene in pIL-2/IgG1 encodes a 484 residue protein, of which the first 20 amino acids (the natural leader segment of IL-2) should be cleaved prior to secretion (17). Thus, the processed protein (464 amino acids) should have a m.w. of approximately 51,000. There is a single asparagine-linked glycosylation site in the CH2 exon of the gene, which occurs naturally in human IgG1 (27). Thus the chimeric gene would be expected to encode a processed, glycosylated molecule with a m.w. of approximately 54,000. This prediction is in agreement with that experimentally determined for the molecule secreted by the 87.20.12 transfectant. The presence of both IL-2 and IgG1 determinants on the same molecule verifies its chimeric nature. The IL-2/IgG1 molecule is secreted by the cell as a disulfide-linked homodimer, presumably possessing the two disulfide bonds that normally link the hinge regions of the H chain.

The chimeric IL-2/IgG1 molecule retains the functional activity of both the IL-2 and Ig portions of the molecule. The N-terminal IL-2 moiety binds IL-2R-positive cells as demonstrated by indirect immunofluorescence. The level of fluorescent intensity observed with IL-2/IgG1 is less than that observed with mAb directed against the human or mouse p55 chain of the IL-2R (Fig. 3). This is most likely due to the excess p55 present on the cell surface that is not associated with p75 to form a high affinity IL-2R (28), but would be detected by the antibodies. The chimeric molecule can also mediate the proliferation of an IL-2-dependent T cell line with a specific activity indistinguishable from human RIL-2. This latter result implies that the affinity of IL-2/IgG1 for the IL-2R is very similar to that of IL-2.

The maintenance of Ig effector function in IL-2/IgG1 indicates that the Ig molecule can tolerate a structurally

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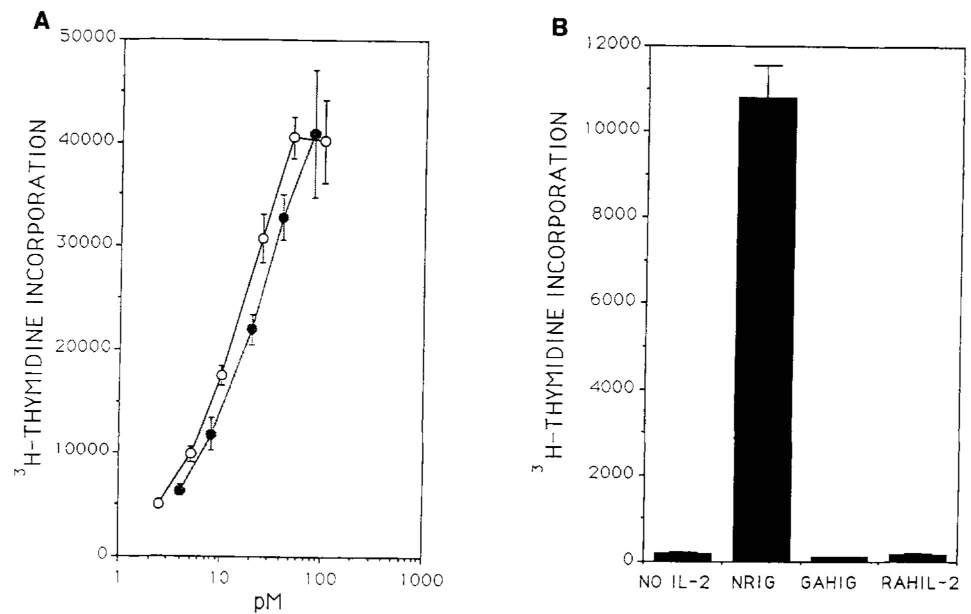

Figure 4. A, varying concentrations of human rIL-2 (●) or IL-2/IgG1 (○) were assayed for the ability to stimulate the proliferation of CTLL cells, as measured by the incorporation of [^{3}H]thymidine. The SE of each determination is presented on the graph. *B*, a 100-pM solution of IL-2/IgG1 was preincubated with normal rabbit IgG agarose (*NRIG*); goat anti-human IgG agarose (*GAHIG*); rabbit anti-human IL-2 agarose (*RAHIL-2*); prior to analysis for the ability to stimulate the proliferation of CTLL cells. The level of proliferation of CTLL cells in the absence of exogenous IL-2 is indicated on the far left (*NO IL-2*).

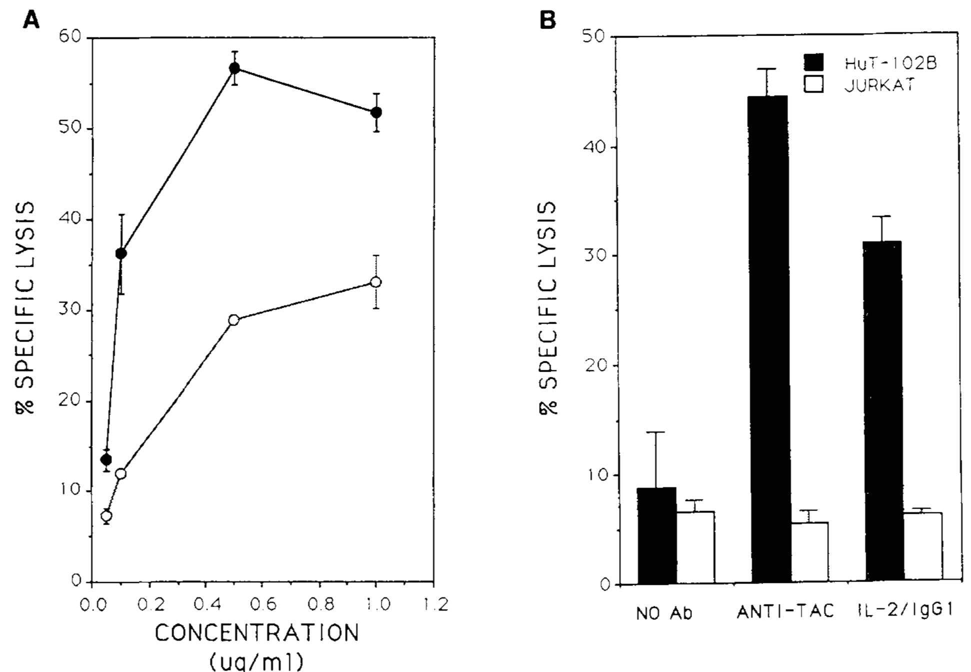

Figure 5. A, varying concentrations of the murine anti-Tac mAb (●) or IL-2/IgG1 (○) were assayed for the ability to mediate complement-dependent lysis of ^{51}Cr-labeled HuT-102B cells. *B*, anti-Tac and IL-2/IgG1 were assayed for the ability to mediate complement-dependent lysis of the human T cell lines HuT-102B and Jurkat. The concentration of each reagent was 5 μg/ml. The SE for all determinations are presented on the graphs.

different moiety in place of the variable domain with little or no impairment of function. Previous constructs with the human CD4 molecule attached to Ig constant regions have demonstrated differing levels of Ig effector function. A CD4/IgG chimeric containing all three constant region domains did not bind C1q (13), whereas a similar molecule lacking the CH1 domain did exhibit detectable C1q binding (14). In both instances, the analysis was limited only to the interaction of C1q with the constant region, and did not address the capacity of the molecules to actually mediate the lysis of the appropriate cells. The ability to bind C1q does not always correlate with the capacity to mediate specific cell lysis (29). More recently, a CD4/Ig chimeric has exhibited ADCC activity in a 20-h assay (15).

The ability of the Ig constant region in IL-2/IgG1 to mediate complement-dependent cell lysis and ADCC demonstrates that these effector functions can be retained in molecules where the binding specificity is imparted not by Ig variable regions, but rather by a ligand of choice. Furthermore, the domain responsible for binding specificity need not be a member of the Ig superfamily. I propose that such molecules be referred to as immunoligands.

The successful replacement of an Ig variable region with the structurally disparate IL-2 and retention of Ig effector function reveals the potential to create a variety of immunoligands in which the binding specificity is non-Ig in nature (e.g., hormone, lectin, peptide, or other ligand). Such agents could have therapeutic potential if their binding specificity is unique to a neoplasia or other tissue characteristic of a disease state.

An additional potential application of immunoligands derives from the maintenance of IL-2 activity in the

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chimeric molecule. IL-2, like several other small therapeutically useful molecules, exhibits a short serum half-life (30). Immunoligands consisting of small biologically active molecules coupled to Ig isotypes that mediate effector function poorly (or a specifically modified Ig constant region devoid of effector function) should exhibit an increased serum half-life, and thus could serve as a more stable form of the original molecule.

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