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15. Substrate activation was observed for BAP at concentrations greater than $5 \times 10^{-4} M$ PNPP in tris at low ionic strength. Below this value a linear relation was observed for the Lineweaver-Burk plot. At 1*M* tris the plot is linear over a wide substrate range. In contrast to the behavior of BAP, TAP shows no substrate activation.
16. We thank Drs. H. Inouye and J. Beckwith for providing the original alkaline phosphatase plasmid and *E. coli* strains used in this work; Drs. D. Kendall, P. Model, and M. Russel for advice; and J. F. Harris, J. Marrinan, D. Levitan, K. Howard, and E. Radziejewski for technical assistance. Supported in part by a postdoctoral fellowship AM 07122-02 (S.E.R.) from the National Institutes of Health. This report is dedicated to the memory of Dr. Kazys Martinkus.

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Specific Immunosuppression by Immunotoxins Containing Daunomycin

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Daunomycin, when conjugated with a targeting antigen by an acid-sensitive spacer, remains inactive at the intravascular *p*H of 7 but becomes active after cleavage within the acidic lysosomal environment of the target cell. This observation made it possible to construct cytocidal compounds that caused antigen-specific suppression of murine lymphocyte function. When daunomycin was coupled to the hapten conjugate of ovalbumin by an acid-sensitive *cis*-aconityl group, it caused hapten-specific impairment of immunocompetence in murine B lymphocytes in vitro and in vivo. Furthermore, the response by T lymphocytes to concanavalin A in vitro was selectively eliminated by a conjugate between daunomycin plus the acid-sensitive spacer and a monoclonal antibody specific for T cells.

THE SPECIFIC TARGETING OF CYtocidal drugs to selectively eliminate undesirable cells has met with only limited success, particularly in vivo (*1*). Recently, various toxins, including ricin, have proved to be specifically cytotoxic in vitro and, in a few cases, in vivo after conjugation to antibodies raised against cell surface markers (*1–4*). The clinical potential of immunotoxins includes the immunotherapy of cancer and the alleviation of various immunoregulatory disorders and allograft rejection. For example, clones of B lymphocytes may be selectively eliminated by the specific binding of the antigen-toxin conjugate to cell surface immunoglobulin receptors. Such studies have involved the specific deletion in vitro of B lymphocytes by ricin that had been conjugated to antigens, including self antigens such as acetylcholine receptors and thyroglobulin (*5–7*).

Unfortunately, the use of toxic peptides such as ricin raises the serious problem of nonspecific toxicity, a formidable obstacle for application in vivo (*4*). For this reason, we have used daunomycin (DM) as a cytocidal drug in conjunction with a targeting hapten-coupled protein (*8*). When directly linked to a protein carrier by reaction with an amino group, DM tends (in some cases) to lose its cytocidal potential (*9*). This problem was overcome through the introduction of an acid-sensitive spacer such as a *cis*-aconityl group (CA) between carrier and drug (*10*). The carrier–(CA-DM) conjugate remained stable at the intravascular *p*H of 7 but once endocytosed by the target cell, the drug was cleaved in the intralysosomal acid environment and entered into the cytoplasm. Intercalation of DM appears to make template DNA unavailable to DNA and RNA polymerases (*11, 12*).

We report here that trinitrophenol (TNP)-, 4-hydroxy-3-iodo-5 nitrophenylacetic acid (NIP)-, and fluorescein (FLU)-ovalbumin (OVA) conjugates, when coupled to CA-DM, are capable of inducing hapten-specific immunosuppression in murine lymphocytes in vitro and in vivo. We also demonstrate that conjugates between a T-cell–specific monoclonal antibody (anti-Thy 1.2) and CA-DM selectively suppress the proliferative response by murine spleen cells to the T-cell mitogen concanavalin A (Con A). This effect probably reflects the selective binding of the drug-antigen conjugate to target B or T lymphocytes bearing surface receptors for the hapten or anti-Thy 1.2, respectively.

To prepare CA-DM, a modified version of the method of Shen and Ryser (*10, 13*)

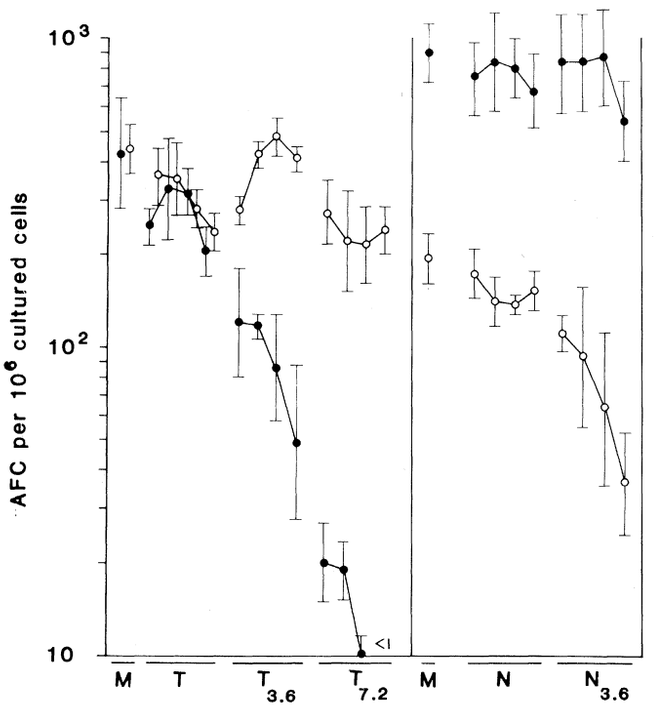

Fig. 1. TNP- or NIP-specific immunosuppression induced in vitro with TNP-OVA-(CA-DM) or NIP-OVA-(CA-DM). In each group, interconnected symbols represent treatment of splenocytes (4×10^6 cells per milliliter) for 2 hours at 4°C with the indicated conjugate at 50, 100, 250, and 500 μg/ml. M, medium alone; T, TNP_7-OVA; $T_{3.6}$, TNP_7-OVA-$(CA\text{-}DM)_{3.6}$; $T_{7.2}$, TNP-OVA-$(CA\text{-}DM)_{7.2}$; N, NIP_3-OVA; $N_{3.6}$, NIP_3-OVA-$(CA\text{-}DM)_{3.6}$. The treated cells were washed and challenged with TNP-Ba (closed circles) or NIP-Ficoll (open circles) in vitro for 4 days. Mean numbers of antibody-forming cells (AFC) ± standard deviation are indicated on the ordinate.

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was used. Twelve milligrams of *cis*-aconitic anhydride were dissolved in 3 ml of dioxane at room temperature and added dropwise to an ice-cold solution of DM (12 mg in 6 ml of distilled water) while stirring and keeping the *p*H at 9 by addition of 0.5*N* NaOH. After 15 minutes, the *p*H was adjusted to 7 with 0.5*N* HCl and the reaction was allowed to proceed for 1 hour. Protein carrier–(CA-DM) conjugates were prepared as described (*10*) by mixing reactants on ice while stirring. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] was added and the reaction mixture was kept at *p*H 7 for 3 hours. Conjugates were dialyzed overnight against distilled water at *p*H 7 and further purified by low-pressure liquid chromatography. The above protocol was also applied to the coupling of butacaine hemisulfate (BC). Hapten-protein conjugates were prepared as described (*14*). Conjugation ratios were expressed in terms of numbers of TNP, FLU, NIP, DM, or BC per 100,000 daltons of OVA. For tissue culture, 5×10^5 murine spleen cells were suspended in 0.3 ml of serum protein–free DSI medium (Quadra Logic Technologies Inc., Canada) supplemented with $5 \times 10^{-5}M$ mercaptoethanol. Cultures were set up as previously described (*14*). Cells that produced antibodies to TNP, NIP, or FLU were enumerated by means of hapten-coupled sheep red blood cells (*15, 16*) as targets for hapten-specific immunoglobulin M (IgM) antibody. Hapten-coupled *Brucella abortus* (Ba) (*17*) were used for immunization in vitro (14×10^8 organisms per culture) and in vivo (1×10^{10} organisms per animal given intravenously). CBA/CaJ male and female mice, 8 to 12 weeks old (Ellerslie Animal Farm, University of Alberta, Canada), were used.

The numbers of hapten-specific antibody-forming cells were markedly reduced relative to control groups in lymphocyte populations that had been incubated with TNP- or NIP-OVA-(CA-DM) before challenge with appropriate antigens (Fig. 1). All in vitro experiments were repeated at least three times, yielding similar results. Each treatment group consisted of four to five separate cultures. Values reported are means ± standard deviation. A mixture of free DM and antigen lacked immunosuppression specificity, which indicates the importance of having the drug physically linked to the antigen. Treatment of splenocytes with a mixture of 500 μg of TNP_7-OVA and increasing amounts of free DM (0, 0.1, 1, and 10 μg/ml) resulted in increased suppression of the TNP-specific response (672 ± 95, 459 ± 87; 72 ± 48; and 8 ± 9 antibody-forming cells per 10^6 cultured cells, respectively) as well as suppression of the NIP-specific response (332 ± 107, 52 ± 18, 6 ± 3, and <1 antibody-forming cells per 10^6 cultured cells, respectively). In contrast, treatment of spleen cells with TNP_7-OVA-$(CA\text{-}DM)_{7.2}$ at 250 μg/ml (containing 10 μg of coupled DM per milliliter) resulted in suppression of only the TNP-specific response (Fig. 1). The suppressive effect of the immunotoxin depended on its concentra-

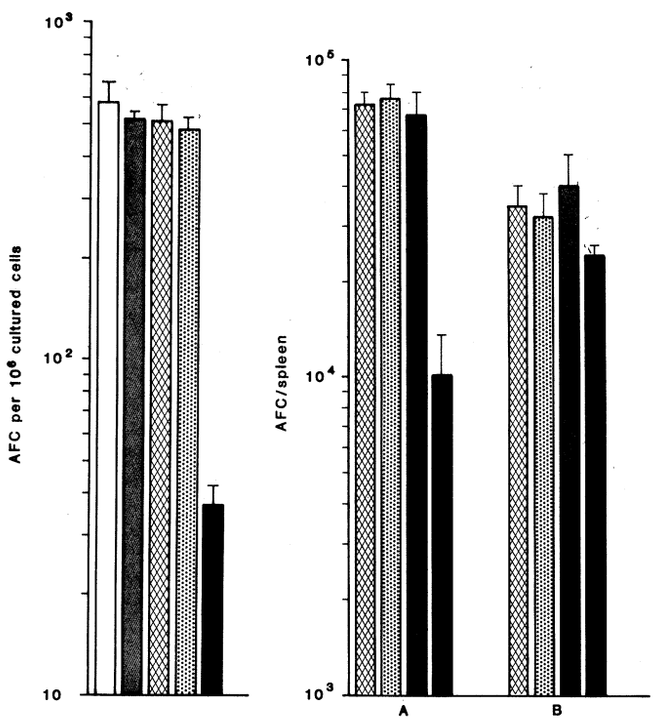

Fig. 2 (left). Effectiveness of TNP-OVA-CA-DM depends on the attachment of DM to the protein carrier by the acid-sensitive spacer molecule CA. Treatment of splenocytes with medium alone (open bar), TNP_3-OVA-CA (hatched bar), TNP_3-OVA-DM (crosshatched bar), TNP_3-OVA-$(CA\text{-}BC)_{5.4}$ (dotted bar), or TNP_3-OVA-$(CA\text{-}DM)_{5.4}$ (solid bar); final concentration of conjugate was 500 μg/ml. The mean numbers of cells forming antibodies (AFC) to TNP ± standard deviation are shown. Fig. 3 (right). TNP-specific immunosuppression induced in vivo with TNP-OVA-CA-DM. Mice in groups of five were each injected intravenously with saline (crosshatched bars), 600 μg of TNP_3-OVA-CA (dotted bars), TNP_3-OVA-$(CA\text{-}BC)_{7.2}$ (hatched bars), or TNP_3-OVA-$(CA\text{-}DM)_{7.2}$ (solid bars). The mice were simultaneously challenged with TNP-Ba and FLU-Ba 24 hours later. Mean number of cells forming antibodies (AFC) to TNP ± standard deviation are shown in (A) and to FLU in (B).

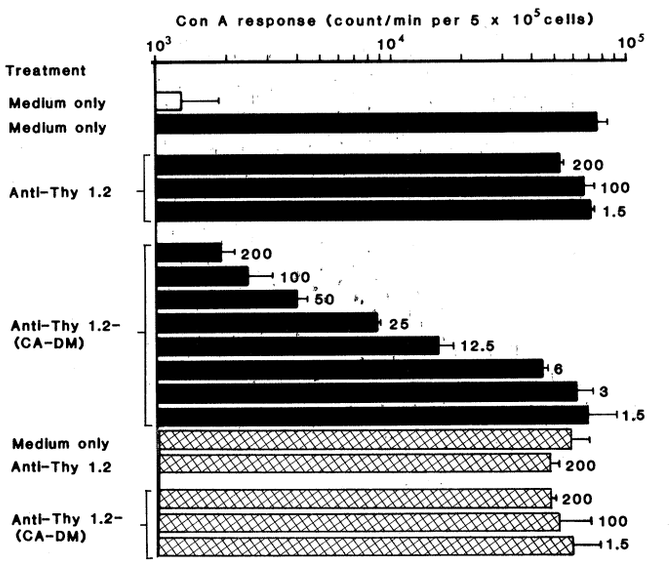

Fig. 4. Immunosuppression of T-cell response to Con A by anti-Thy 1.2–(CA-DM). Spleen cells (5×10^5) were incubated at 4°C for 2 hours with anti-Thy 1.2 or with anti-Thy 1.2 (CA-DM) at different concentrations, washed, and cultured for 3 days in the presence of Con A (2 μg/ml) or LPS (10 μg/ml). Uptake of [^{3}H]thymidine is expressed ± standard deviation for the responses by unstimulated cells (open bar), by cells stimulated with Con A (solid bars), or by cells stimulated with LPS (hatched bars). Numbers at the top of the bars represent concentrations in micrograms per millimeter. The amount of DM contained in anti-Thy 1.2 at 200 μg/ml was 10 μg. The concentration of conjugated DM required to suppress the Con A response by 50 percent was 0.3 μg/ml. Free DM at 0.1 μg/ml suppressed the Con A response by 50 percent. This relationship between concentrations of conjugated and free DM does not mean that conjugated DM is less effective than free DM at the cellular level since, during incubation of the cells at 4°C, a much larger amount of free DM is expected to enter the cell (presumably by diffusion) than of conjugated DM. In the latter case, only amounts of drug actually bound to the cell surface will be endocytosed into the cell. The antibody used in these experiments was of the IgM class (*19*).

tion and on the ratio between the CA-DM group and the carrier (Fig. 1). Finally, since the antibody responses induced by hapten-coupled Ba or Ficoll were independent of antigen-specific T cells, it is likely that our immunotoxin acts directly on B cells.

We next assessed the relevance of the acid-sensitive spacer CA to the immunosuppressive potency of the antigen-DM conjugate. Normal splenocytes were incubated with medium alone, TNP_3-OVA-CA, TNP_3-OVA-$DM_{5.4}$, TNP_3-OVA-$(CA\text{-}DM)_{5.4}$, or or a haptenated conjugate in which DM was replaced by butacaine (BC) (a compound of similar molecular weight as DM but without cytotoxic activity). After incubation, the cells were washed and challenged in vitro with TNP-Ba. Immunosuppression resulted from DM-antigen treatment only if the antibiotic was linked to the carrier protein by the acid-sensitive spacer group (Fig. 2).

To determine the potency of our antigen–(CA-DM) conjugate as a specific immunosuppressant in vivo, we injected CBA/CaJ mice intravenously with saline or with various conjugates. After 24 hours, the mice were simultaneously challenged with both TNP-Ba and FLU-Ba and assayed for AFC 5 days later. The conjugate containing CA-DM was markedly and specifically immunosuppressive without visibly affecting the health of the animals during the experiment (Fig. 3).

We next extended our studies to the use of monoclonal antibody as the target-specific carrier of CA-DM. Spleen cells were incubated with anti-Thy 1.2 that had been coupled to CA-DM, for 2 hours at 4°C. The cells were then washed and cultured in the presence of Con A or the B-cell mitogen lipopolysaccharide (LPS). After 3 days, cellular uptake of [^{3}H]thymidine was determined as a measure of DNA synthesis (*18*). At concentrations that completely suppressed the T-cell response to Con A, anti-Thy 1.2–(CA-DM) had no marked effect on the B-cell response to LPS (Fig. 4). Incubation of spleen cells with mixtures of the target-specific carrier and free DM resulted in nonspecific suppression.

Our experiments with antigen as the target-specific ligand indicate that immunosuppression may be obtained not only in vitro, but also in vivo with little or no adverse side effects to the animal. The construction of target-specific immunosuppressive compounds that contain small molecular weight cytocidal drugs may have the potential for human clinical use in bone marrow transplantation and the treatment of autoimmune disease. In our experiments, antigen-specific immunosuppression was obtained in mice that had not previously been exposed to antigen (Fig. 3). In cases where autoantibody is the principal cause of an autoimmune disorder, neutralization by such antibody of an autoantigen-containing immunotoxin may not occur if nonspecific immunosuppression is induced prior to immunotoxin therapy. Autoimmune diseases such as multiple sclerosis, which is thought to be mediated by T lymphocytes, may be particularly suited for therapy with an immunotoxin whose targeting moiety is a monoclonal antibody directed at the appropriate T-cell subset.

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19. In other experiments, we have been able to selectively kill surface Ig-bearing B cells with an IgG-containing immunotoxin. Furthermore, and in confirmation of work by others [J. Gallego, M. R. Price, R. W. Baldwin, *Int. J. Cancer* **33**, 737 (1984)], we have also been able to selectively kill tumor cell lines with an IgG-containing immunotoxin. This renders it unlikely that the data presented in Fig. 4 are due to properties specifically associated with the Ig-isotype used as the targeting conjugant in the immunotoxin.

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Antigens Induced on Erythrocytes by *P. falciparum*: Expression of Diverse and Conserved Determinants

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Red blood cells that are infected with the malaria parasite *Plasmodium falciparum* express new antigens on their surface. In a study of these antigens in the erythrocytes of naturally infected children in the Gambia, an antibody-mediated agglutination assay revealed an extreme degree of antigenic diversity. Serum samples from each of ten children in the convalescent stage of malaria infection reacted with infected cells from the same child but generally not with infected cells from the other children. The Gambian children's erythrocytes also expressed shared determinants: sera from Gambian adults often reacted with the surface of infected cells from all of the children and were shown by adsorption and elution experiments to contain antibodies that recognized several isolates. Conserved determinants exposed on infected erythrocytes may be important for development of antimalarial immunity either naturally or through vaccination.

ASEXUAL BLOOD STAGES OF THE MALARIA PARASITE induce numerous changes in the morphology, functional properties, and antigenicity of the surface membrane on the parasitized erythrocyte (*1*). In the simian parasite *Plasmodium knowlesi*, a malarial protein was shown to be inserted into the erythrocyte membrane and responsible for the phenomenon of antigenic variation at the surface of these infected cells (*2*). Antigens specific for the surface of infected erythrocytes could be potential targets for vaccination against malaria if they expressed determinants that were conserved among isolates from different geographical regions. An early immunological and electron microscopy study (*3*) provided evidence for such an antigenically conserved determinant in *Plasmodium falciparum*–infected erythrocytes from the mon-

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