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Immunology

Prevention of autoimmune lysis by T cells with specificity for a heat shock protein by antisense oligonucleotide treatment

ULRICH STEINHOFF*†, ULRICH ZÜGEL*, ANGELA WAND-WÜRTTENBERGER*, HARTMUT HENGEL‡, RUDI RÖSCH§, MARTIN E. MUNK*, AND STEFAN H. E. KAUFMANN*¶

*Department of Immunology, ‡Department of Virology, and §Section of Polymers, University of Ulm, Albert-Einstein-Allee 11, D-89070 Ulm, Germany

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ABSTRACT **T lymphocytes with specificity for the bacterial heat shock protein (hsp) 60 recognize stressed host cells, thus possibly promoting pathogenesis of certain infectious and autoimmune diseases. Here, we show that autoimmune destruction of stressed Schwann cells and macrophages by cytotoxic T lymphocytes raised against mycobacterial hsp60 can be inhibited by the use of hsp60-specific antisense oligodeoxynucleotides (A-ODNs). The inhibitory effect of hsp60 A-ODNs was specific because lysis of murine cytomegalovirus-infected host cells by virus-specific cytotoxic lymphocytes was not affected. Immunoblot analysis and immunoprecipitation studies suggest that different forms of stress increase hsp60 synthesis in Schwann cells and that this neosynthesis is reduced by hsp60 A-ODNs. These findings (*i*) provide evidence for participation of endogenous hsp60 in the recognition of stressed host cells by mycobacterial hsp60-crossreactive T cells and (*ii*) suggest the feasibility of inhibiting autoimmune reactions by target-cell treatment with specific A-ODNs.**

Heat shock proteins (hsp) are among the most ubiquitous polypeptides in the biosphere, being equally essential for numerous biological functions of prokaryotic and eukaryotic cells (1, 2). hsp are not only functionally highly conserved but are also structurally conserved, and certain hsp—e.g., hsp60—share >50% sequence homology between the prokaryotic and the human cognate (3–5). T cells with specificity for regions shared by the bacterial and human hsp60 have been identified, and cytotoxic T lymphocytes (CTL) with specificity for bacterial hsp60 recognize stressed host cells in the absence of bacterial hsp peptides (6–10). These findings suggest that hsp-reactive T cells provide a link between the antibacterial immune response and the pathogenesis of infectious and autoimmune disease. hsp synthesis is increased in stressed host cells, and recognition of such conserved antigens of undistinguishable origin may represent a general threat in various autoimmune and infectious diseases. For example, destruction of stressed Schwann cells by CTL specific for mycobacterial hsp60 indicates that this mechanism contributes to the nerve damage, which is a major pathologic complication of leprosy (11). In previous studies we have developed an *in vitro* model for analyzing CTL-mediated lysis of stressed macrophages and Schwann cells (6–11). Using this model, we now employed hsp60 antisense oligodeoxynucleotides (A-ODNs) (*i*) to assess the direct involvement of hsp60 as target antigen of stressed cells for CTL reactive with mycobacterial hsp60 and (*ii*) to inhibit autoimmune target-cell destruction.

MATERIALS AND METHODS

Antibodies and hsp60. The following antibodies were used in this study: 28-14-8S [mouse IgG monoclonal antibody (mAb) anti-H-2D^b, provided by G. F. Hämmerling, Heidelberg], II-13 [mouse IgG mAb anti-hsp60, provided by R. Gupta, Hamilton, Ontario (12)], and polyclonal rabbit anti-hsp60 serum (13). To obtain hsp60, cultures of recombinant *Escherichia coli* strain M1103 expressing the *Mycobacterium bovis* hsp60 were sonicated and ammonium sulfate-precipitated at 20–55% saturation. The precipitate was purified by anion-exchange chromatography. Purified hsp60 was then denaturated in urea, dialyzed, and digested overnight with trypsin (Sigma) (9).

Cell Cultures. Schwann cell cultures were established from 1- to 2-day-old C57BL/6 mice, as described (11). In short, sciatic nerves were isolated and treated with 0.25% trypsin (Sigma) and 0.25% collagenase (Boehringer Mannheim) in phosphate-buffered saline for 45 min at 37°C and afterward with DNase I at 10 μg/ml (Sigma) for another 45 min at 37°C. Cells were mechanically dissociated by repeated pipetting and cultured in Iscove's Dulbecco's modified medium/1% fetal calf serum/laminin at 2 μg/ml (BRL). Bone marrow macrophages (BMM) were generated from 6- to 8-week-old C57BL/6 or BALB/c mice cultured in Teflon film bags (Heraeus, Hanau, Germany) in a serum-free medium and used after 9 to 10 days of culture (14). Cloned hsp60-reactive CTL were propagated in the presence of spleen cells with cyclic restimulations with tryptic fragments of the mycobacterial hsp60 (8). These CTL not only lysed syngeneic target cells pulsed with the tryptic digest of mycobacterial hsp60 but also lysed interferon γ (IFN-γ)-stimulated host cells in the absence of mycobacterial hsp60 (8, 9). Murine cytomegalovirus (MCMV)-specific CTL were derived from BALB/c mice primed with the recombinant vaccinia virus MCMV-ie1-Vac and restimulated with the immediate-early protein pp89-derived nonapeptide YPHFMPTNL (15, 16).

Induction of hsp60 by Various Stress Stimuli. Established Schwann cell or BMM cultures were stressed to express hsp60 by IFN-γ stimulation for 36 hr, infection with viable *Mycobacterium leprae* (100 bacteria per cell) for 24 hr, or exposure to 42°C–44°C for 30 min. The viability of cells was controlled by microscopic inspection and the trypan blue exclusion test.

Immunoblot Analysis of Stressed Cells. Stressed cells were lysed in lysis buffer (0.05 μM Tris/1 μM leupeptin/1 μM pepstatin/100 μM leupeptin/0.5% Triton X-100), separated on SDS/7% PAGE (equivalents of 5×10^5 cells per lane), and transferred to nitrocellulose by semidry blotting. To ensure usage of equivalent amounts of proteins in each setup, they were analyzed in parallel in Coomassie-stained gels and in a bichinonic acid (BCA) protein assay (Pierce). For immuno-

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Abbreviations: CTL, cytotoxic T lymphocyte(s); hsp, heat shock protein(s); mAb, monoclonal antibody; IFN-γ, interferon γ; MHC, major histocompatibility complex; MCMV, murine cytomegalovirus; A-ODN and S-ODN, antisense and sense oligodeoxynucleotide, respectively; BMM, bone marrow macrophages.

†Present address: Department of Experimental Immunology, University Hospital, Schmelzbergstrasse 12, 8091 Zürich, Switzerland.
¶To whom reprint requests should be addressed.

blot the blotted nitrocellulose was blocked overnight in 3% skim milk in TBS (Tris-buffered saline, pH 7.5). For development we used the mAb II-13 at high dilution (1:50,000). Signal detection was performed with an ECL detection kit (Amersham) as described in the manual. Signals were quantified by scanning densitometry (LKB Ultrascan XL, enhanced laser densitometer).

[^{35}S]Methionine Labeling, Immunoprecipitation, and Autoradiography of Stressed Schwann Cells. Cells were stressed by IFN-γ stimulation, *M. leprae* infection, or heat, cultured in methionine-free medium for 1 hr, and subsequently labeled with [^{35}S]methionine at 100 μCi/ml (1 Ci = 37 GBq) for 2 hr. After careful washing, an equivalent number of cells (3×10^5) were lysed in 200 μl of lysis buffer. Lysates were incubated overnight with 10 μg of rabbit anti-hsp60 antiserum at 4°C (13). For precipitation of antigen–antibody complexes, 50 μl of protein A–Sepharose was added for 1 hr and centrifuged (5 min at 13,000 × *g*). After washing, protein was eluted from beads by boiling with reducing sample buffer and then subjected to SDS/10% PAGE. After drying, the gel was exposed for autoradiography.

hsp60-Specific A-ODNs. Thiophosphate-modified A-ODNs and control sense-ODNs (S-ODNs) specific for eukaryotic hsp60 were synthesized according to the phosphoramidite method (17). The following sequences were used: A-ODNs, 5′-AGCATTTCTGCGGGG-3′; S-ODNs, 5′-CCCCGCAGA-AATGCT-3′. Analysis of the homology between the synthesized oligomer and the primate sequences present in the GenBank data bases (release 73.0) of the Genetics Computer Group sequence analysis software package revealed that the synthetic oligomers were fully complementary only to their own specific mRNA. Efficiency of A-ODN uptake was measured by incubating cells with 20 μM acridine-modified A-ODNs for 20 and 40 hr, respectively (Appligen, Heidelberg). Before analysis on an Epics V cytometer (Coulter), cells were washed thoroughly to remove extracellular A-ODNs. For inhibition of hsp60 neosynthesis, 5 μM A-ODNs were added to the culture medium, and cells were incubated for at least 8 hr before further treatments.

CTL Assays and Target-Cell Treatment. CTL activities were determined by using a standard protocol. Target cells (Schwann cells or BMM) were cultured in 96-well flat-bottom microtiter plates at a density of 10^4 cells per well. Adherently growing cells were incubated with A-ODNs or S-ODNs for 8 hr before IFN-γ stimulation, infection with *M. leprae*, or infection with MCMV. Subsequently, cells were labeled in microtiter plates with $Na^{51}CrO_4$ at 2.5 μCi/well for 19 hr (Schwann cells) or 2 hr (BMM). After careful washing, effector CTL were added at the ratios indicated. For peptide pulsing, cells were incubated with a tryptic digest of hsp60 (10 μg per well) directly before the assay. Target cells selectively express early gene products of MCMV only when infected in the presence of phosphonoacetic acid at 250 μg/ml (15, 16). Data are expressed as follows: percentage of specific ^{51}Cr release = 100 × (experimental − low control)/(high control − low control). Symbols represent mean values of at least three determinations with SD <10%; experiments were repeated twice with comparable results.

RESULTS

Efficient Uptake of hsp60 A-ODNs by Schwann Cells and BMM and Lack of Influence on Major Histocompatibility Complex (MHC) Class I Surface Expression. Phosphorothioate-modified A-ODNs spanning the initiation codon of the hsp60 (−10 to +5) were used to prevent neosynthesis of the endogenous hsp60. Phosphorothioate-modified ODNs are particularly useful due to their increased stability when compared with unmodified ODNs (18). Efficient uptake of the hsp60 A-ODNs by Schwann cells and BMM was verified by cytofluorimetry using an acridine derivative of the A-ODNs (Fig. 1*A*). Murine Schwann cells do not constitutively express detectable amounts of MHC class I gene products on their surface but become MHC class I$^+$ after IFN-γ stimulation (11). This IFN-γ-induced expression of MHC class I molecules was not affected by the A-ODNs as assessed by cytofluorimetry (Fig. 1*B*).

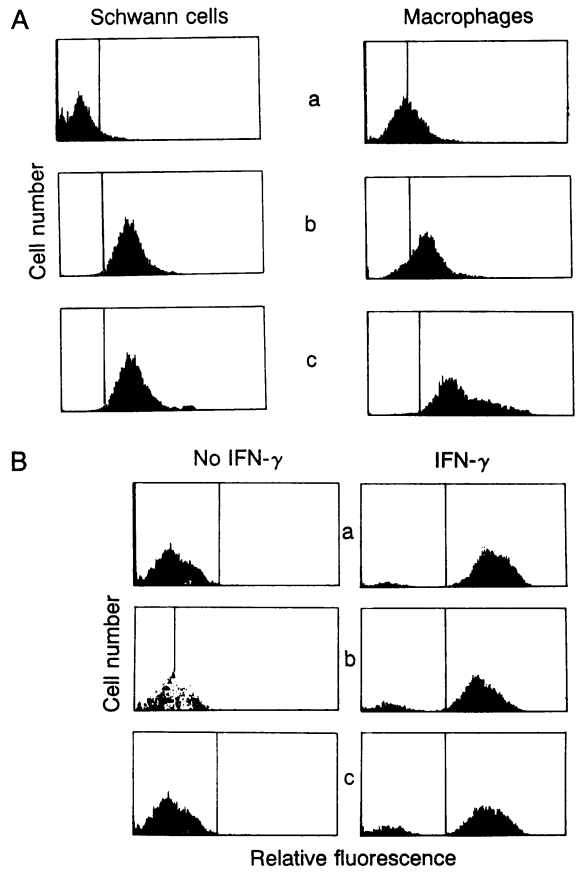

FIG. 1. A-ODN uptake and lack of influence on IFN-γ-induced MHC class I expression. (*A*) A-ODN uptake by Schwann cells and BMM. Cells were left untreated (*a*) or incubated with 20 μM acridine-modified A-ODNs for 20 hr (*b*) or 40 hr (*c*). Before fluorescence-activated cell sorter analysis, cells were washed thoroughly to remove extracellular A-ODNs. Scales on each graph: *x* axis, 0–256 cells; *y* axis, 0–256 channels. (*B*) IFN-γ-induced MHC-class I expression in Schwann cells is not affected by A-ODNs. Schwann cell cultures were left untreated (*a*) or incubated with 10 μM (*b*) or 20 μM (*c*) A-ODNs. Eight hours later, cells were stimulated with IFN-γ(500 units/ml) for 36 hr. Cells were stained with the anti-H-2D^b mAb followed by secondary F(ab′)$_2$ anti-mouse IgG-fluorescein isothiocyanate-labeled antibody. MHC class I expression was monitored by cell cytometry analysis. Results are expressed as in *A*.

Evidence for Increased hsp60 Synthesis in Stressed Host Cells and Its Inhibition by hsp60 A-ODNs. BMM were stressed by heat or IFN-γ stimulation, and then lysates were subjected to immunoblot analyses with the hsp60-specific mAb II-13. Fig. 2 shows a marked increase of hsp60 expression under the various stress conditions. Densitometric scanning reveals a 15-fold increase of the IFN-γ-induced hsp60 signal. Similar results were obtained with stressed Schwann cells using immunoprecipitation with polyclonal anti-hsp60 antibodies (data not shown). Next, we assessed the inhibitory effects of A-ODN treatment on hsp60 synthesis under the aforementioned conditions. Pretreatment with the hsp60 A-ODNs markedly reduced hsp60 expression in Schwann cells as detected by the hsp60-specific mAb II-13 in immunoblot analysis (Fig. 3). The hsp60 A-ODNs reduced the IFN-γ-induced hsp60 signal by 40%, as revealed by scanning

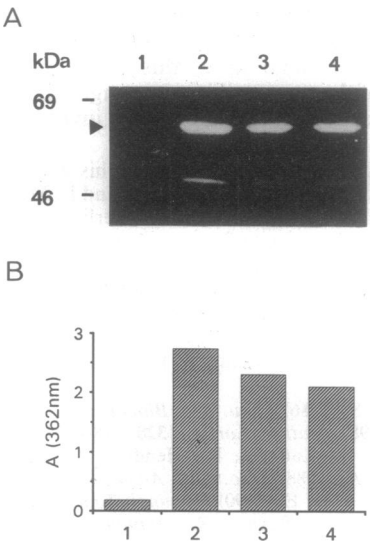

FIG. 2. Immunoblot analysis of hsp60 induction in BMM by different stress stimuli. BMM were either left untreated (lane 1), stimulated with recombinant IFN-γ at 500 units/ml for 36 hr (lane 2), or exposed to 44°C (lane 3) or 42°C (lane 4) for 30 min. Six hours after heat exposure, an equivalent number of BMM were lysed and analyzed by immunoblot (*A*). The 60-kDa bands were quantified in absorbance units/mm² by densitometric scanning (*B*). The hsp60 signal (arrowhead) was increased by IFN-γ (15 fold), by 44°C (12 fold), or by 42°C (11 fold).

densitometry. A similar reduction by hsp60 A-ODNs was observed by immunoprecipitation (data not shown). The corresponding S-ODNs used as controls did not affect hsp60 neosynthesis in these stressed cells (data not shown). We conclude that hsp60-specific A-ODNs interfere with the neosynthesis of hsp60 in macrophages and Schwann cells.

Evidence That hsp60 A-ODNs Specifically Interfere with CTL-Mediated Lysis of Stressed Cells. The effect of A-ODN treatment on Schwann cell lysis by hsp60-reactive CTL was analyzed by using a cloned CD8 CTL line raised against mycobacterial hsp60 that crossreacts with stressed host cells (8–10). These CTL effectively lysed IFN-γ-stimulated Schwann cells in the presence or absence of mycobacterial hsp60 peptides (Fig. 4*A*). On the other hand, IFN-γ-induced MHC class I expression was required for presentation of hsp60 peptides by Schwann cells to these CTL (refs. 9 and 10 and data not shown). Importantly, treatment of Schwann cells with hsp60 A-ODNs 8 hr before IFN-γ stimulation markedly reduced lysis of stressed cells, and this effect was reversed by addition of exogenous hsp60 peptides (Fig. 4*B*). Thus, only processing and presentation of endogenous hsp60 were prevented by hsp60 A-ODN treatment, whereas induction of MHC class I gene products, as well as presentation of exogenous hsp60 peptides by MHC class I molecules, was not affected. Simultaneous incubation of Schwann cells with hsp60 A-ODNs and IFN-γ did not interfere with lysis, indicating that uptake of hsp60 A-ODNs and effective blocking of hsp60 transcripts are necessary for the inhibition of Schwann cell recognition by the hsp60-specific CTL line (Fig. 4*A*). Similar effects were seen when BMM were used as target cells (Fig. 5*A*). In contrast, lysis of BMM infected with MCMV by a virus-specific CD8 CTL line was not affected by hsp60-specific A-ODNs (Fig. 5*B*) (15, 16). Recognition of MCMV-infected target cells by these CTL was based on the functional presentation of a virus-derived peptide under early conditions (15, 16). These experiments suggest that (*i*) hsp60 A-ODN treatment selectively interfered with the destruction of stressed cells by hsp60-specific CTL and (*ii*) hsp60 is not involved in presentation of endogenously derived MCMV peptides.

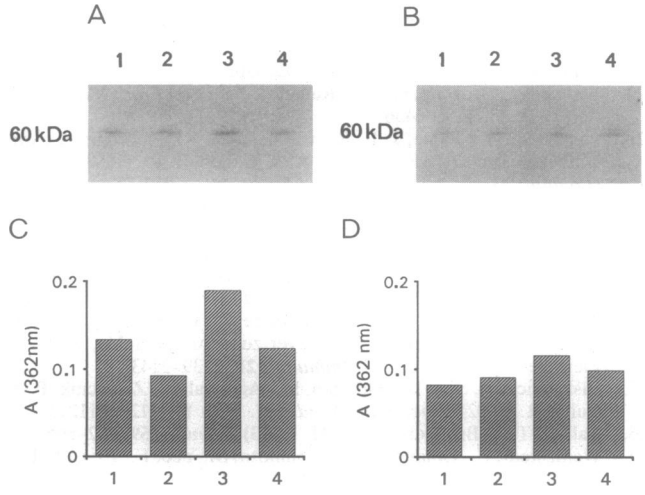

FIG. 3. A-ODN treatment inhibits hsp60 neosynthesis in stressed Schwann cells. Immunoblot analyses of lysates from stressed Schwann cells in the absence (*A*) or presence (*B*) of A-ODN. A-ODN treatment was done 8 hr before infection with viable *M. leprae* (100 bacteria per cell) for 36 hr (lane 1) or before stimulation with 500 units of IFN-γ (lane 3) or 50 units of IFN-γ (lane 4). A-ODN treatment of unstimulated Schwann cells served as control (lane 2). Equal numbers of Schwann cells were lysed and subjected to PAGE. Bands are quantified by densitometric scanning (*C* and *D*). A-ODN treatment reduced the hsp60 signal in controls by 3%; in cells infected with *M. leprae* reduction was by 38% and in cells stimulated with 50 units or 500 units of IFN-γ reduction was 39% or 20%, respectively.

DISCUSSION

Our experiments show that hsp60 A-ODNs (*i*) inhibit hsp60 expression caused by various stress stimuli including IFN-γ and, as a corollary, (*ii*) interfere with CTL-mediated specific destruction of IFN-γ-stressed cells. The hsp60-A-ODN effect was specific because it did not affect IFN-γ-induced MHC class I surface expression in Schwann cells or recognition of

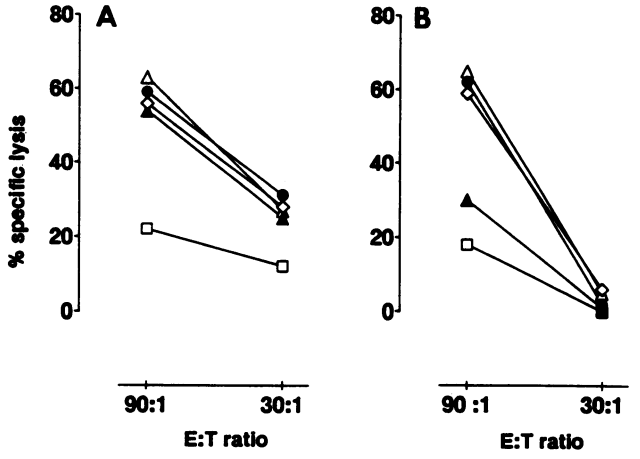

FIG. 4. Lysis of stressed Schwann cells by hsp60-specific CTL is inhibited by A-ODN treatment. Target cells were incubated with A-ODNs and simultaneously stimulated with IFN-γ (*A*) or incubated with A-ODNs 8 hr before initiation of IFN-γ stimulation (*B*). Schwann cells were treated as follows: □, untreated and unstimulated; △, untreated and IFN-γ-stimulated; ◇, S-ODN-treated plus IFN-γ-stimulated; ▲, A-ODN-treated plus IFN-γ-stimulated; ●, A-ODN-treated, IFN-γ-stimulated plus hsp60 peptides-pulsed. Hsp60-specific CTL were used as effector cells. Symbols represent mean values of at least three determinations with SD <10%; experiments were repeated twice with comparable results. E:T, effector cell/target cell.

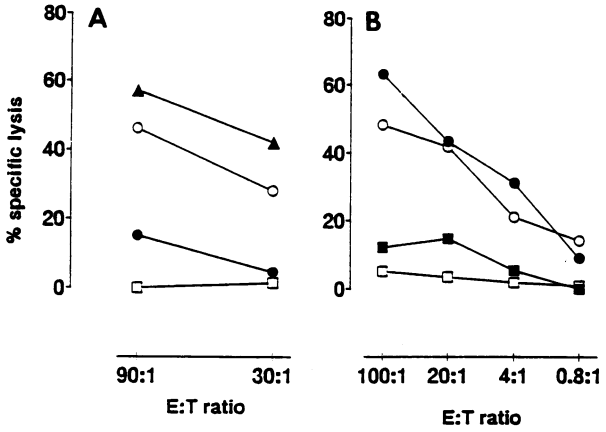

FIG. 5. Evidence for the specificity of A-ODN treatment in target-cell lysis. (*A*) C57BL/6 BMM were incubated with A-ODN 8 hr before the following treatments: □, untreated; ○, IFN-γ-stimulated; ●, A-ODN-treated plus IFN-γ-stimulated; ▲, A-ODN-treated, IFN-γ-stimulated pulsed with tryptic peptides of hsp60. hsp60-specific CTL were used as effector cells. (*B*) BALB/c BMM were incubated with A-ODN 8 hr before infection with MCMV: □, untreated; ○, infected with MCMV; ■, A-ODN-treated; ●, A-ODN-treated plus infected with MCMV. MCMV-specific CTL were used as effector cells. Symbols represent mean values of at least three determinations with SD <10%; experiments were repeated twice with similar results. E:T, effector cell/target cell.

MCMV-infected target cells by specific CD8 CTL. We take these findings as evidence that the self-epitope recognized by the CD8 T cells raised against mycobacterial hsp60 is derived from hsp60 in the target cells. However, the possibility remains, that self-hsp 60 is selectively involved in processing of unrelated self-epitopes, but not of MCMV-derived epitopes. In these experiments we used hsp60-mediated destruction of stressed Schwann cells as a model for nerve damage in leprosy. Evidence has been presented that hsp60 and hsp70 represent target antigens for α/β and/or γ/δ T cells in various autoimmune diseases, including adjuvant arthritis of rats and type I diabetes of non-obese diabetic mice as well as juvenile arthritis, reactive arthritis, multiple sclerosis, and systemic lupus erythematosus of humans (19–24). Therefore, our findings suggest a general feasibility of A-ODN application toward characterization and down-regulation of hsp-related autoreactivity. Although A-ODNs have been successfully used not only *in vitro* but also in selected *in vivo* models (25–27), we are aware that controlled delivery and uptake of macromolecules to specific tissues and cells still present problems. However, substantial progress has been achieved by using ligand-conjugated liposomes (28) or by exploiting natural endocytosis pathways—using, e.g., the folate receptor (29).

Earlier attempts to interfere with autoimmune damage by T cells have accomplished inhibition of T-cell subpopulations through phenotype or T-cell receptor isotype and were independent of the T-cell epitope specificity (30, 31). This strategy, however, is relatively nonspecific, for it affects not only T-cell clones responsible for autoimmune damage but also unrelated T-cell clones that coincidently express the same phenotype or T-cell receptor but have a different antigen specificity. Alternatively, inhibition of autoimmune damage has been attempted by competitively inhibiting interactions between the T-cell receptor and its epitope using peptide analogs (32). Success of this strategy is complicated by the HLA polymorphism, necessitating application of different peptides to prevent target-cell recognition by T cells in different individuals. In contrast, blocking the neosynthesis of potential autoantigens with specific A-ODNs, as described here, excludes T cells of irrelevant epitope specificity but expressing the same phenotype and T-cell receptor isotype. At the same time, this strategy interferes with recognition of the responsible autoantigen independent of the fine epitope specificity of the T cells involved.

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