Epicutaneous Application of CpG Oligodeoxynucleotides with Peptide or Protein Antigen Promotes the Generation of CTL

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Immunostimulatory oligodeoxynucleotides (ODN) are effective adjuvants in the induction of humoral and cellular immune responses when administered parenterally with antigen. The skin has recently become a target organ for the design of non-invasive vaccine technologies. Using ovalbumin (OVA) as a model antigen, we demonstrate that the application of ODN sequences to tape-stripped skin promotes the induction of potent cytotoxic T lymphocyte (CTL) responses to co-administered peptide. Induction of peptide-specific CTL required the presence of CpG motifs within the ODN. CTL afforded tumor protection against a tumor expressing an immunodominant OVA CTL epitope. CTL could also be induced to whole protein administered onto the skin. Differential CpG sequence activity was noted with respect to the induction of CTL to epicutaneous protein with an ODN sequence containing a poly-G motif having an optimal effect. Peptide-specific CTL could be detected in the peripheral blood as early as 6 d after a single immunization. These results highlight the potential of the bare skin as a route for vaccine development and indicate an important role for immunostimulatory ODN as adjuvants to generate functional CTL with the help of the skin immune system.

Key words: adjuvant/CTL/oligodeoxynucleotides/skin/tumor
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Vaccination is a cost-effective method for promoting health. Most vaccination protocols, however, rely on parental administration of antigen entailing the use of needles with the attendant costs of trained personnel and the risk of blood-borne pathogen transmission. Further, as the development and availability of new vaccines proceeds, children are exposed to needles more frequently and this association with pain and discomfort may decrease compliance. There has thus been interest in the development of non-invasive routes of delivery. In particular, the use of the skin as target organ for vaccine design (Babiuk *et al*, 2000; Hammond *et al*, 2001; Partidos *et al*, 2001) has been spurred by the fact that epidermal dendritic cells (DC), termed Langerhans cells, are among the most efficient presenters of antigen to the immune system (Romani *et al*, 1989; Celluzzi and Falo, 1997) and are abundant in the skin (Bergstresser *et al*, 1980). Recent interest in the use of the skin as a vaccination site has followed the observations that repeated epicutaneous exposure to protein antigens could induce dominant Th2 T cell and antibody responses (Saloga *et al*, 1994). Glenn and colleagues have demonstrated that cholera toxin (CT) (Glenn *et al*, 1998a) and other bacterial toxins (Glenn *et al*, 1999) can be used as transcutaneous adjuvants for protein immunization through the skin. Repeated application of proteins on the skin in this manner was found to result in the generation of serum and mucosal IgG and IgA antibodies capable of mediating protective immunity against mucosal (Glenn *et al*, 1998b) and systemic (Glenn *et al*, 1999) toxin challenge. Induction of antigen-specific $CD4^+$ T cell responses with a Th2 bias in this manner has also been demonstrated (Beignon *et al*, 2001; Hammond *et al*, 2001).

Cytotoxic T lymphocytes (CTL) are crucial in the mammalian immune defense against tumors and viruses (Bangham and McMichael, 1989; Boon *et al*, 1994). A limitation of current vaccine strategies, however, is the inefficient priming of CTL (Steinman and Pope, 2002). Unmethylated CpG dinucleotides flanked by certain bases (CpG motifs) have been shown to be immunostimulatory (Krieg *et al*, 1995; Sato *et al*, 1996; Klinman *et al*, 1997). Synthetic oligodeoxynucleotides (ODN)-bearing immunostimulatory CpG motifs have been shown to potentiate CTL responses to co-administered peptides or proteins emulsified in IFA and to promote survival in response to tumor challenge (Krieg *et al*, 1995; Sato *et al*, 1996; Klinman *et al*, 1997). These motifs also allow the induction of CTL responses to specific peptides in the absence of additional adjuvant (Vabulas *et al*, 2000; Miconnet *et al*, 2002). Herein, we explore the possibility that ODN can be used epicutaneously to promote the generation of CTL to co-administered peptide or protein antigen. We find that ODN sequences can be added, in an epicutaneous immunization protocol, to either the model foreign protein ovalbumin (OVA) or the immunodominant class I MHC-restricted peptide derived from OVA to induce potent epitope-specific

Abbreviations: CT, cholera toxin; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FMOC, 9-fluorenylmethoxy carbonyl; MHC, major histocompatibility complex; ODN, oligodeoxynucleotide; OVA, ovalbumin; PE, phycoerythrin; SFC, spot-forming cells

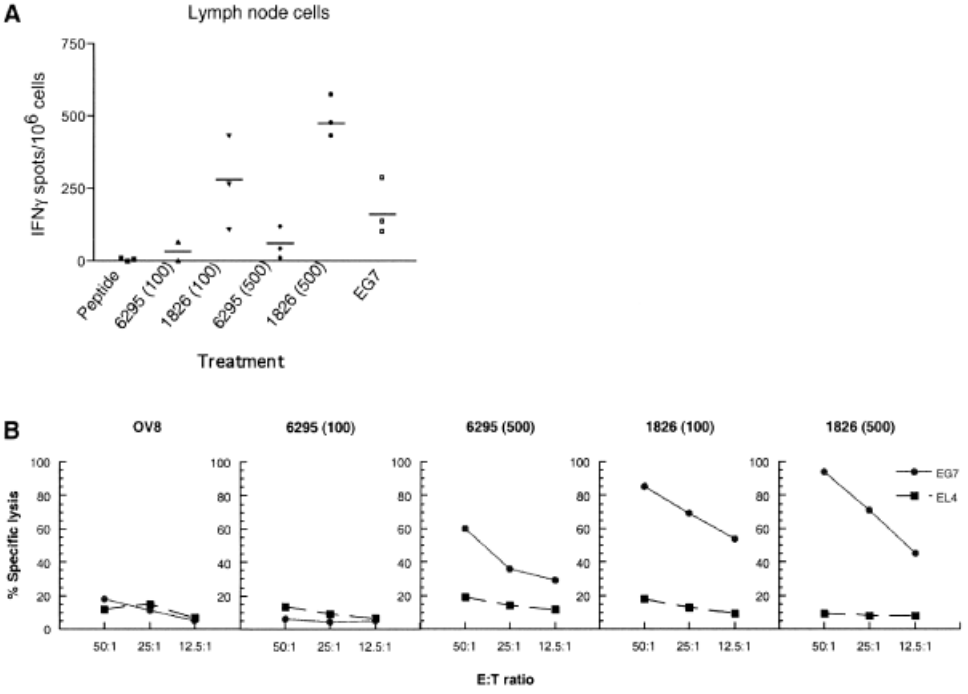

Figure 1
Effect of oligodeoxynucleotides on the induction of CTL by epicutaneous immunization of mice with peptide antigen. Mice were immunized with 25 μg OV8 peptide applied to tape-stripped ear skin and boosted 1 wk later. Immunization was completed in the presence of either 100 or 500 μg ODN (6295 or 1826) with the dose indicated in parenthesis. Mice injected with a tumor cell line transfected with ovalbumin (EG7) were used as a positive control. One wk after boosting, lymph node cells and splenocytes were harvested and assayed for the presence of CTL precursors using ELISpot (*Panel A*) or standard CTL assay (*Panel B*). (*Panel A*) Scatter gram of peptide-specific spot-forming cells (IFNγ-spots) detected in the lymph nodes of immunized mice. (*Panel B*) Chromium release assay: mice immunized as in *Panel A*. Splenocytes were re-stimulated *in vitro* prior to assay for cytolytic activity. Data points correspond to the average of triplicates from individual mice and varied less than 10%. These assays are representative of three independent experiments.

CTL responses that demonstrate protective activity in an induced tumor model.

Results

ODN promote the generation of CTL to epicutaneously administered peptide Contact hypersensitivity responses to epicutaneously administered haptens such as dinitrofluorobenzene are mediated by the priming and effector functions of $CD8^+$ T cells (Bour *et al*, 1995). Further, epidermal Langerhans cells can induce potent antigen-specific CTL when pulsed with class I MHC-restricted epitopes and adoptively transferred into naïve hosts (Celluzzi and Falo, 1997). We thus sought to induce CTL by the epicutaneous administration of peptide. To study epicutaneous CTL priming we chose to use OVA as a model system as the immunodominant epitope of OVA (SIINFEKL) presented in the context of the K^b MHC molecule is well characterized (Rotzschke *et al*, 1991). This system was also chosen as the growth of OVA-bearing tumor cells (EG7; EL-4 thymoma cells transfected with OVA cDNA) (Moore *et al*, 1988) allows an *in vivo* read-out of the efficacy of CTL induction.

Tape-stripping is a simple method to remove the epidermal barrier of the stratum corneum. We therefore immunized mice immediately following tape-stripping with either 25 μg OV8 peptide alone or with 100 or 500 μg of immunostimulatory ODN. We used a sequence well known to induce Th1 immunity, ODN-1826 (Chu *et al*, 1997). We also tested a sequence, ODN-6295, that is complementary to the initiation codon sequence of the proto-oncogene c-myc and contains a 5′ CpG motif that induces strong Th1 responses in mice (Mui *et al*, 2001). To quantitate the generation of CTL *in vivo*, we used an ELISpot assay that detects antigen-specific IFNγ release by activated CTL (Fig 1*A*). Mice were injected with live EG7 cells to generate CTL as a positive control. Treatment with peptide alone resulted in few OV8-specific SFC (6 ± 3 SFC per 10^6 in the lymph nodes and 6 ± 5 SFC per 10^6 in the spleen, $n = 3$). Immunization in the presence of ODN-1826, however, resulted in a significant increase in OV8-specific SFC in the lymph nodes at a concentration of either 100 μg (268 ± 90, $n = 3$, $p < 0.05$ compared with peptide alone) or 500 μg (495 ± 40, $n = 3$, $p < 0.001$ compared with peptide alone). To determine if CTL precursor cells were capable of inducing mature, functional CTL, splenocytes were re-stimulated *in vitro* with irradiated EG7 cells and then tested for CTL activity in standard chromium release assays (Fig 1*B*). CTL activity against the OVA-bearing tumor, EG7, but not against the control EL4 was generated following epicutaneous immunization in the presence of 500 μg ODN-1826 and less cytolytic activity was noted with 100 μg ODN-1826. Although few peptide-specific SFC were detected in the draining lymph nodes of mice immunized with ODN-6295 and peptide (Fig 1*A*), CTL activity could be generated from splenocytes of mice immunized with 500 μg ODN-6295 (Fig 1*B*). It should be noted that CTL responses were never observed when ODN in PBS was applied with antigen to the skin without barrier disruption by tape-stripping (data not shown) or when peptide was applied without ODN. Thus, epicutaneous application of peptide to tape-stripped skin, in the presence of ODN can induce CTL to the applied peptide. The response is ODN concentration-dependent. Different ODN sequences have varying ability to enhance CTL generation epicutaneously to co-administered peptide.

Class I MHC tetramers are novel reagents that allow the rapid quantification of CTL frequencies immediately *ex vivo* (Altman *et al*, 1996). The fraction of epitope-specific CTL detected by *ex vivo* staining of blood lymphocytes for tumor-specific CTL has been shown to correlate with the

fraction of such cells present in the spleen and lymph nodes (Miconnet *et al*, 2002). As mice immunized with ODN-1826 and peptide showed an optimal CTL response, we used K^b-OVA tetramers to further study the peripheral blood of mice immunized with OV8 and ODN-1826. To study the requirement for a CpG sequence in the immunostimulatory effect of the ODN we also used ODN-1982, which has the identical base composition as ODN-1826 without the CpG motif (Fig 2). To determine if the ODN applied had a systemic immunostimulatory effect or a local adjuvant effect ODN was applied to the contralateral ear, at a site distant to the peptide immunogen. Mice were immunized once, boosted at 7 d, and bled at 12 d. The peripheral blood of mice immunized with ODN-1982 and OV8 peptide contained a low frequency of OV8-specific CTL (0.05% of $CD8^+$ T cells), similar to un-immunized controls. With ODN-1826, roughly 20-fold more OV8-specific CTL were detected (1.09% of $CD8^+$ T cells). Application of ODN-1826 to the contralateral ear completely negated the adjuvant effect indicating that there is no systemic immune activation or adjuvant effect following the topical application of 500 μg ODN-1826. Functional analysis of CTL precursors present in the spleen using ELISpot assay confirmed the results obtained by blood tetramer analysis demonstrating a 3-fold increase in peptide-specific SFC when ODN-1826, but not control ODN-1982 was co-applied with peptide immunogen (Fig 2*B*). The application of ODN-1826 resulted in activation of $CD11c^{hi}$ DC in the draining lymph nodes when evaluated at 20 h as evidenced by increased CD80 and CD86 expression when compared with DC from mice treated with ODN-1982 (Fig 2*C*).

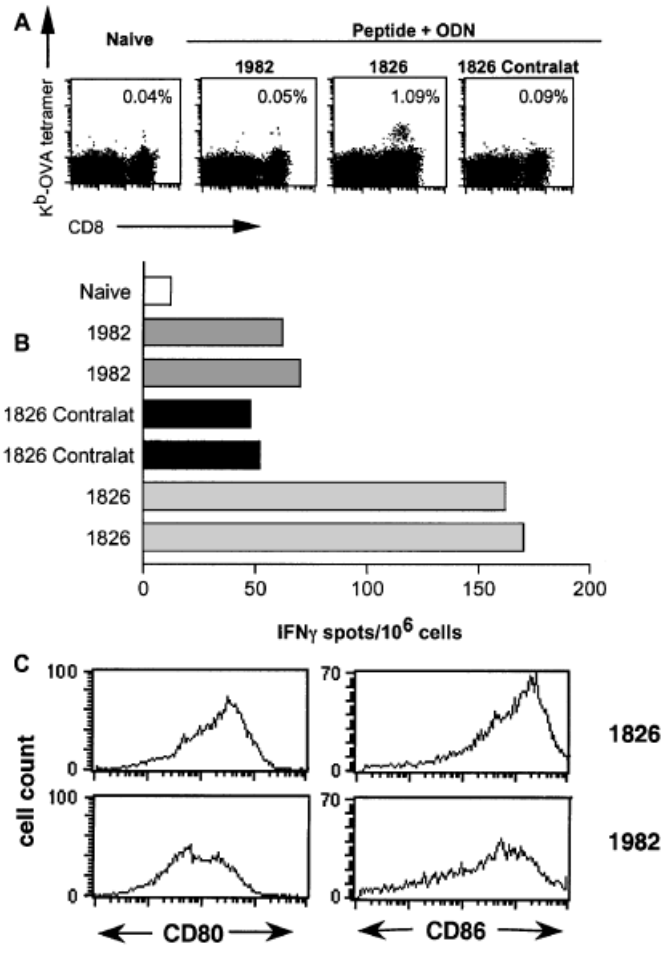

Figure 2
Effect of oligodeoxynucleotide sequence and location of application on the generation of CTL to epicutaneously administered peptide. Mice were immunized with 25 μg OV8 peptide on tape-stripped ears in the presence of the 500 μg dose of ODN-1826 or with the control ODN-1982, boosted in similar fashion at 1 wk (day 7) and bled at 12 d following the initial immunization. ODN was applied either with the peptide immunogen or separately on the contralateral ear (Contralat.). (*Panel A*) Representative dot-plots of peripheral blood lymphocytes demonstrating CD8 expression and K^b-OVA tetramer staining. The numbers represent the percent K^b-OVA-specific $CD8^+$ T cells among all $CD8^+$ T cells detected. (*Panel B*) Bar graph of peptide-specific IFNγ-spot-forming cells (IFNγ-spots) detected in the spleens from individual mice immunized as in *Panel A*. (*Panel C*) Draining lymph node dendritic cell ($CD11c^{hi}$) expression of CD80 and CD86 20 h following application of either ODN-1826 or ODN-1982 to the ear. These assays are representative of two independent experiments.

Epicutaneous immunization with peptide and ODN affords tumor protection As an *in vivo* read-out of the efficacy of CTL induction, we used the inhibition of the growth of OVA-bearing thymoma tumor cells (EG7). Immunization with OVA and adjuvant can diminish and prevent the growth of EG7 tumor cells injected subcutaneously (Porgador *et al*, 1997). CT has been used as an epicutaneous adjuvant to enhance antibody responses to epicutaneously administered proteins (Glenn *et al*, 1998a). CT has also been used to promote the generation of CTL responses after mucosal immunization (Porgador *et al*, 1997). We have determined that CT can enhance the induction of CTL to epicutaneously administered peptide (Kahlon *et al*, 2003). To compare the ability of immunostimulatory ODN and CT to provide tumor immunoprophylaxis, groups of eight mice were immunized epicutaneously. Groups of four mice were bled on day 12 to document the presence of OV8-specific CTL *ex vivo*. Mice then received 5×10^6 EG7 tumor cells subcutaneously and the growth of resultant tumors was monitored for 3 wk (Fig 3). Mice immunized epicutaneously with OV8 peptide in the presence of the ODN-1826 (0.82% ± 0.19% of $CD8^+$ T cells, $n = 4$, $p < 0.05$) demonstrated a significant increase in OV8-specific CTL in the peripheral blood when compared with sham immunized mice (0.11% ± 0.01%). Mice immunized with either peptide alone (0.19% ± 0.03%, $n = 4$), peptide with ODN-6295 (0.27% ± 0.08%, $n = 4$) or CT (0.48% ± 0.04%, $n = 4$) did not have statistically significant differences in the frequency of OV8-specific CTL in the peripheral blood when compared with the other groups. Concordant with this, there was a significant inhibition of tumor growth only in mice immunized with OV8 and ODN-1826 (Fig 3*B*, $p = 0.0006$). To confirm that the inhibition of tumor growth was secondary to a peptide-specific CTL response and not due to an antigen non-specific mechanism, mice were immunized with ODN-1826 and the tumor-expressed OV8 peptide or an irrelevant K^b-restricted peptide (2C) and compared with mice immunized with OV8 peptide and control ODN-1982 adjuvant. Significant numbers of OV8-specific CTL were only found in the blood of animals immunized with OV8 and ODN-1826 (Fig 3*C*). Similarly, tumor growth inhibition was only noted in mice treated with

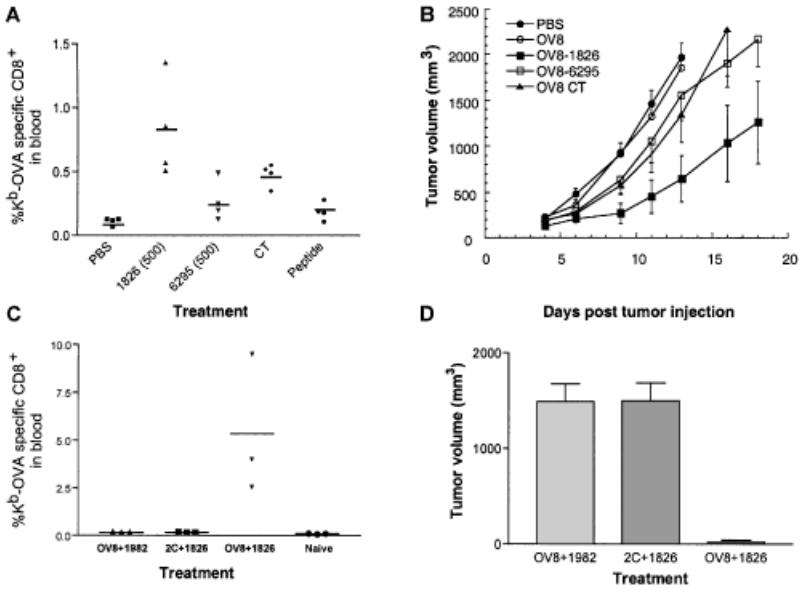

Figure 3
Effect of epicutaneous immunization on tumor immunoprophylaxis and correlation with *ex vivo* enumeration of antigen-specific CTL among peripheral blood lymphocytes. Mice were immunized epicutaneously and boosted once with OV8 peptide alone or with either 25 μg cholera toxin (CT) or 500 μg ODN (6295 or 1826). Mice treated with PBS were used as a negative control. Mice were bled on day 12, prior to inoculation with an OVA-bearing tumor. The percent of OV8 peptide-specific CTL among the $CD8^+$ T cells in peripheral blood lymphocytes was determined in individual mice using a OVA-K^b tetramer. A scatter plot of the results for four mice per group is represented in *Panel A*. The means were significantly different ($p = 0.019$, the Kruskal–Wallis test) with a *post hoc* comparison between PBS and 1826 (500) demonstrating a significant difference ($p < 0.01$, Dunn's multiple comparison test). Tumor volumes were then measured serially in groups of eight mice and are depicted as a function of time in *Panel B*. Error bars represent SEM. The differences between groups were significant ($p = 0.0006$) with a *post hoc* comparison using a Bonferroni adjustment revealing a significant difference between PBS *versus* OV8-1826 ($p = 0.002$) and between OV8-1826 *versus* OV8 ($p = 0.002$). (*Panel C*) Mice were immunized with OV8 peptide or the irrelevant K^b-restricted control peptide 2C together with ODN-1826, or with OV8 peptide and the control ODN-1982. Mice were bled on day 12, prior to tumor inoculation. A scatter plot of the results of three mice per group is shown. There was a significant difference between groups ($p = 0.02$, the Kruskal–Wallis test) with a significant difference between OV8 + 1826 *versus* naïve ($p < 0.05$) and OV8 + 1826 *versus* OV8 + 1982 ($p < 0.05$). Mice were then inoculated with an OVA-bearing tumor. Tumor volumes were then measured at day 14. The average of tumor sizes in individual mice are indicated in *Panel D* with error bars indicating SEM. There was a significant difference between OV8 + 1982 *versus* OV8 + 1826 ($p = 0.002$, unpaired *T* test) and 2C + 1892 and OV8 + 1826 ($p = 0.001$, unpaired *T* test). Results are representative of two independent experiments.

OV8 peptide and ODN-1826 adjuvant (Fig 3*D*). Thus epicutaneous immunization with peptide in the presence of ODN-1826 induces functional CTL *in vivo* and the presence of these CTL correlates with an inhibitory effect on cognate peptide-bearing tumor growth.

ODN sequences differentially promote CTL induction to an epicutaneously administered protein or peptide Epicutaneous immunization using whole proteins has been demonstrated to result in the generation of antibody responses when accompanied by the application of bacterial-derived adjuvant. CTL priming to epicutaneously applied peptide following tape-stripping has been demonstrated previously but epicutaneous protein administration has only resulted in negligible CTL responses. We have recently demonstrated that the co-administration of CT with whole protein to tape-stripped skin can induce robust CTL responses (Kahlon *et al*, 2003). We therefore sought to determine if co-administration of immunostimulatory ODN sequences with OVA would enable local protein uptake and processing to result in priming of OV8-specific CTL. To this end, mice were immunized and boosted once with 500 μg OVA protein and ODN-6295 or ODN-1826, or their control sequences ODN-6300 or ODN-1982, respectively. Following intradermal immunization with peptide and ODN-1826, the frequency of peptide-specific CTL peaks at 7 d (Miconnet *et al*, 2002). We determined the frequency of OV8-specific $CD8^+$ T cells after a single immunization and a boost (Fig 4). Surprisingly, ODN-6295 generated more CTL than ODN-1826 when using a protein as an immunogen and we could detect OV8-specific $CD8^+$ T cells 6 d after a single epicutaneous immunization. A second "booster" immunization at 7 d enhanced the number of CTL in the peripheral blood when analyzed at day 12. To confirm the differential activity of ODN-6295 on a protein *versus* peptide immunogen, mice were immunized with either 500 μg OVA protein ($\sim$ 12 nmol) or 25 μg of OV8 peptide ($\sim$ 24 nmol) and 500 μg of ODN (either ODN-6295, or ODN-1826). To determine if functional CTL precursors were generated, splenocytes were then stimulated once *in vitro* and tested in a chromium release assay (Fig 4*B*). Epicutaneously administered peptide or whole protein could induce potent CTL activity. Again, the optimal adjuvant immunostimulatory sequence for OVA protein immunization (ODN-6295) differed from the optimal sequence for OV8 peptide immunization (ODN-1826). Mice immunized with protein and the control ODN (1982 or 6300) did not generate CTL activity (data not shown). Thus, the two ODN sequences we have studied differentially promote CTL induction to an epicutaneously administered protein or peptide.

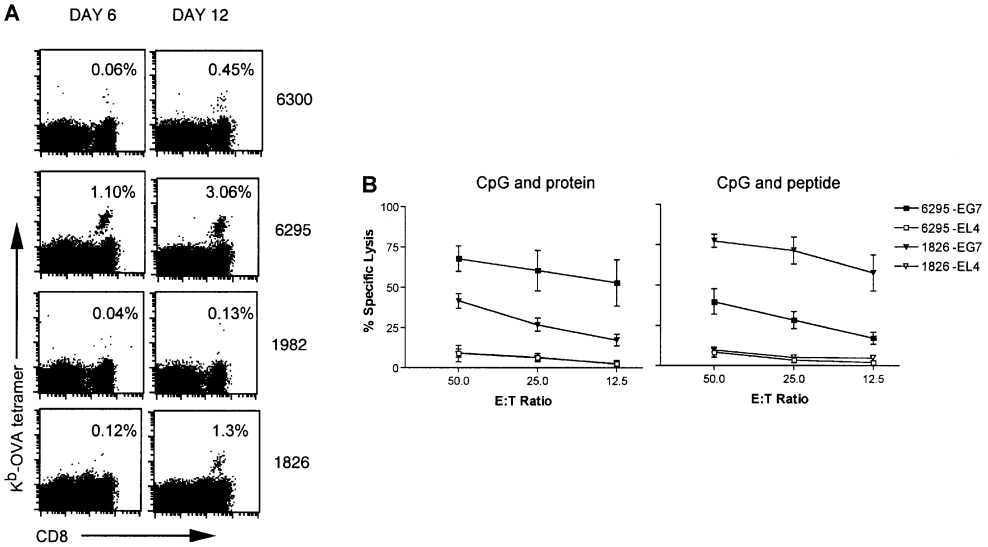

Figure 4
Effect of ODN on the generation of CTL by epicutaneous immunization with either peptide or protein antigen. (*Panel A*) Epicutaneous immunization of mice with protein in the presence of CpG sequences results in the appearance of peptide-specific CTL in the peripheral blood as soon as 6 d after a single epicutaneous immunization and is enhanced following boosting at 1 wk. Mice were immunized epicutaneously with ovalbumin protein (500 μg) and 500 μg ODN (either 6295, 1826 or the respective non-CpG ODN controls, 6300 or 1982). Representative dot-plots of peripheral blood lymphocytes from individual mice after a single immunization on day 6 and again, following a boost 1 wk after immunization (day 12). The numbers represent the percent K^b-OVA-specific $CD8^+$ T cells among all $CD8^+$ T cells detected. The figure is representative of two independent experiments. (*Panel B*) Mice were immunized epicutaneously with either ovalbumin protein (500 μg—left panel) or OV8 peptide (25 μg—right panel) and 500 μg ODN (either 6295 or 1826) as indicated, and boosted once. Splenocytes were harvested 1 wk later, re-stimulated *in vitro* once, and CTL activity against a tumor cell line expressing OVA (EG7) or a control cell line (EL4) was determined. Each data point represents the average CTL activity of three individual mice and error bars represent SEM. The optimal ODN sequence for the induction of CTL to epicutaneous protein immunization (ODN-6295) differed from the optimal sequence for peptide immunization (ODN-1826). The data are representative of three independent experiments.

Discussion

In this study, we demonstrate that topical application of immunostimulatory ODN to tape-stripped skin enhances the generation of CTL to epicutaneously applied peptide or protein antigen. As ODN sequences with CpG motifs are known to activate cutaneous DC (Jakob *et al*, 1998), to enhance their migration from the skin (Ban *et al*, 2000) and to stimulate IL-12 production (Jakob *et al*, 1999), these observations are consistent with the hypothesis that epicutaneously administered protein or peptide antigen is taken up by resident cutaneous DC (either Langerhans cells or dermal DC) and transferred to the draining lymph nodes for presentation to naïve T cells. ODN can exert a moderate adjuvant effect in the generation of humoral immune responses to topically applied toxins such as diphtheria toxoid in the absence of tape-stripping (Scharton-Kersten *et al*, 2000). Epicutaneous peptide administration to tape-stripped skin alone can result in the induction of CTL responses to co-administered antigen (Seo *et al*, 2000). For effective responses, relatively large amounts of peptide ($\sim$ 100 nmol applied twice) were required. Tape-stripping removes the stratum corneum, a significant barrier to large molecular weight antigen (protein or peptide) penetration of the skin. Tape-stripping also promotes local DC migration, presumably in part via the release of IL-1 (Nishijima *et al*, 1997). As ODN have a molecular weight significantly in excess of 500, a commonly described size limit for epicutaneous diffusion in the absence of occlusion or barrier perturbation (Bos and Meinardi, 2000), we chose to study the adjuvancy of such ODN when applied to tape-stripped skin. We demonstrate here that the epicutaneous administration of the ODN with peptide or protein antigen promotes the rapid and robust induction of CTL. CTL can be detected *ex vivo* 6 d after a single administration of antigen. These CTL have *in vivo* activity as detected by their ability to inhibit cognate-antigen expressing tumor growth. Peptide-specificity of the inhibition of tumor growth confirms that the adjuvant effect of the ODN results in the generation of functional CTL and that tumor growth inhibition is not due to other forms of ODN-mediated immune stimulation such as NK cell activation. Although CT (at 25 μg or $\sim$ 0.3 nmol, a dose limited by toxicity) is also able to promote CTL priming to epicutaneous antigen following tape-stripping (Kahlon *et al*, 2003), the ODN-1826 (at 500 μg or $\sim$ 80 nmol, with no toxicity observed) was significantly more effective in generating CTL and in promoting tumor immunoprophylaxis.

ODN-bearing CpG sequences initiate intracellular signaling via the TLR9 receptor (Hemmi *et al*, 2000). Macrophages, B cells, and DC are known to express these receptors. Although keratinocytes have been shown to internalize ODN (Noonberg *et al*, 1993), they are not known to express the TLR9 receptor. Thus epidermal or dermal DC, are the most likely target cells of these reagents following epicutaneous administration. The upregulation of the DC co-stimulatory molecules CD86 and CD80 in draining-lymph nodes by epicutaneous CpG ODN (1826) is consistent with this (Fig 2*C*). Interestingly, an ODN sequence originally designed for c-myc antisense therapy demon-

strated a differential adjuvant activity when compared with ODN-1826 in peptide and protein immunization. Although this sequence was only minimally effective in promoting immune responses to co-administered peptide, it demonstrated enhanced activity, when compared with ODN-1826 in inducing responses to co-administered protein (Fig 4). Thus sequence-specific effects can be induced by ODN when applied onto the skin. Indeed, ODN have been classified into Type A and Type B, based on sequence and putative target cell type (Krieg, 2002). For example, the presence of poly-G motifs at the 5′ and 3′ ends and a phosphodiester palindromic CpG-containing sequence has been shown to preferentially activate NK cells and induce IFNα production in human plasmacytoid DC precursors (Krug *et al*, 2001). Likewise, a CpG sequence containing poly-G motifs was optimal for the regression of melanomas in mice (Ballas *et al*, 2001). In this regard, the ODN-6295 sequence (5′-TAACGTTGAGGGGCAT-3′) contains a CpG sequence (underlined) as well as a four-mer poly-guanine sequence (shaded) not present in ODN-1826 (5′-TCCATGACGTTCCTGACGTT-3′). The presence of plasmacytoid DC in inflammatory skin disease has recently been documented (Farkas *et al*, 2001). Whether the poly-G sequence present in ODN-6295 underlies this ODN's differential ability to enhance responses to epicutaneous protein administration and whether this reflects the activation of a differential subgroup of DC such as plasmacytoid DC remains to be determined. The differential adjuvant effect of the tested ODN sequences on peptide or protein antigen further suggests that the molecular and perhaps cellular pathways by which epitopes from peptide antigen and protein antigen are ultimately displayed on skin-derived antigen-presenting cells that prime CTL differ. Regardless of mechanism, the ability to induce potent CTL responses to epicutaneously administered protein antigens has the advantage of allowing the simultaneous induction of $CD4^+$ T helper responses important in the optimal control of viral infections (Matloubian *et al*, 1994) and tumor growth (Schuler-Thurner *et al*, 2002).

Tape-stripping is a cheap and readily performed procedure that elicits minimal discomfort. It elicits less discomfort than percutaneous needle puncture (Singer *et al*, 1998). Parenteral administration of CpG sequences can be associated with side-effects including extramedullary hematopoeisis (Sparwasser *et al*, 1999), antigen-independent expansion of the naïve and memory T cell compartment (Davila *et al*, 2002), and exacerbation of auto-immune disease (Miyata *et al*, 2000). As we could not detect an immunostimulatory effect following contralateral CpG administration, targeting CpG sequences to cutaneous DC by epicutaneous administration may minimize these risks. Although CT and modified enterotoxins have been used to induce protective humoral and cellular immunity by the epicutaneous route, robust CTL induction has either not been demonstrated (unmodified enterotoxins) or whole CT has been used. The known toxicities of CT continue to make handling cumbersome and are dose limiting. The efficacy of ODN sequences in epicutaneous immunization demonstrated here for both peptide and protein may thus be a significant advance in the design of simple and effective epicutaneous vaccine technologies.

Materials and Methods

Animals and cell lines C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, Massachusetts) and held in a specific pathogen-free environment. All animal experiments were approved by the Animal Care Committee of the University of British Columbia. EL4 thymoma and the E.G7-OVA cells (EL4 transfected to express OVA, here termed EG7) (Carbone and Bevan, 1990) were obtained from the American Type Culture Collection (Manassas, Virginia) and cultured in complete medium (CM) consisting of RMPI 1640 medium (Invitrogen, Burlington, Ont., Canada) supplemented with penicillin G (100 U per mL), streptomycin sulfate (100 μg per mL), 5×10^{-5} M β-mercaptoethanol and 10% fetal calf serum (FCS).

Immunizations, cytotoxicity assay, and tumor growth assay For the induction of CTL, female C57BL/6 mice (routinely 5–6-wk old) were immunized by the application of antigen with or without adjuvant in phosphate-buffered saline (PBS) to tape-stripped ears. For tape-stripping, mice were anesthetized by intraperitoneal injection of ketamine plus xylazine. The ear skin on the dorsal and ventral sides was then tape-stripped 10 times (using Scotch Brand 3710 adhesive tape, 3M, Minnesota). To ensure functional disruption of the epidermal barrier, the surface was next wiped once with acetone. Antigen and adjuvant were then immediately applied. Adjuvants consisted of CT (Sigma, St Louis, Missouri), or one of following phosphorothioate-modified ODN: ODN-1826 (5′-TCCATGACGTTCCTGACGTT-3′) (Chu *et al*, 1997) or the control sequence ODN-1982 (5′-TCCAGGACTTCTCTCAGGTT-3′), ODN-6295 (5′-TAACGTTGAGGGGCAT-3′) (Mui *et al*, 2001) or a control sequence (ODN-6300) for ODN-6295 that maintains the same base composition and four guanosine sequences but disrupts the CpG motif (5″-TAAGCATACGGGGTGT-3′). ODN were purchased from Avecia Biotechnology (Milford, Massachusetts) or Trilink Biotechnologies Inc (SanDiego, California), dissolved in PBS and stored at −20°C until use. ODN were evaluated for endotoxin contamination by suppliers and were considered endotoxin free (<1 EU per mg). Antigen consisted of OVA (OVA V, Sigma, Missouri) or peptide (OV8 or 2C, see below) as indicated. To boost the response, the same procedure was applied to the contralateral ear 1 wk later. Mice were euthanized at day 14. OVA-specific CTL were detected immediately *ex vivo* using fluorescently labeled K^b-OVA class I MHC tetramers (Altman *et al*, 1996). For tetramer analysis of peripheral blood, samples (routinely 100 μL) were obtained by saphenous vein puncture, depleted of red blood cells and analyzed by fluorescence cytometry. To generate CTL for functional assays, 5×10^6 splenocytes depleted of red blood cells were stimulated with 2×10^6 irradiated E.G7-OVA cells (25,000 rad) and cultured in 10 mL CM for 5 d. Human rIL-2 (20 IU per mL) (Pharmingen, California) was added on day 3. Chromium release assays were carried out using ^{51}Cr-labeled E.G7-OVA cells, or EL4 cells as targets. Plates were incubated for 4 h at 37°C. Supernatants were collected and γ-radiation was measured. Specific lysis of target cells was calculated using the formula (release by CTL−medium release)/(detergent release−medium release) × 100. Spontaneous release from target cells was always under 15%. For tumor growth assays, mice were immunized by tape-stripping for 2 consecutive weeks (day 0 and 7). Blood was sampled on day 12. On day 14, EG7 tumor cells (5×10^6) were injected subcutaneously into the left flank of immunized mice. The tumor size was assessed every second day by taking repeated perpendicular measurements using digital calipers (Mitutoyo, Mississauga, Ont., Canada). Tumor volumes were calculated using the standard formula for ellipsoid tumor volumes, (length × width^2)/2 where, by convention, width was taken as the shorter of the two measurements.

Enzyme-linked immunospot (ELISpot) assays A standard cytokine-specific ELISpot assay was used to detect CTL precursor activity *ex vivo* as described (Miyahira *et al*, 1995). Briefly, cells from the skin draining lymph nodes (axillary, cervical, and inguinal)

and splenocytes were incubated in CM with or without 1 μg per mL OV8 peptide and with the addition of 20 IU per mL human rIL-2 for 20 h. As a positive control, cells were stimulated with 25 μg per mL concanavalin A. Cells were seeded onto nitrocellulose 96 microwell plates (Millipore, Bedford, Massachusetts) coated with anti-mouse interferon (IFN)γ capture antibody (R4-6A2, Pharmingen). Biotinylated anti-IFNγ detection antibody (XMG 1.2, Pharmingen) was then used to detect spot-forming cells (SFC) followed by the addition of streptavidin-alkaline phosphatase (Bio-Rad, Hercules, California) and 5-bromo-4-chloro-3 indolyl phosphate/nitroblue tetrazolium substrate (Biorad). Spots were enumerated with the aid of a stereo microscope. Values are expressed as the number of peptide-specific SFC and were obtained by subtracting the number of spots obtained without peptide from those obtained with peptide stimulation per million cells. Values represent the average of duplicate wells.

Cytofluorometric analysis Three-channel fluorescence-activated cell sorting (FACS) analysis was performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, California) using Cell Quest software (Becton Dickinson). Briefly, 200,000 cells were analyzed after staining with appropriate antibodies or fluorochrome-labeled class I MHC tetramer. Phycoerythrin (PE) labeled K^b-OVA tetramers were purchased from Beckman Coulter Immunomics (San Diego, California). Antibodies to CD8α (clone YTS 169.4) were from Cedarlane (Hornby, Canada).

Dendritic cell preparation and analysis To quantitate the expression of co-stimulatory molecules on DC, the ODN (500 μg) were applied to the ears and draining lymph nodes were collected 20 h later. Single-cell suspensions were prepared by treating the lymph nodes of mice with 1 mg per mL of collagenase D (Boehringer Mannheim, Indianapolis, Indiana) supplemented with 40 μg per mL DNAse (Boehringer, Mannheim) and 10 mM EDTA. DC were enriched using anti-CD11c mAb-coated microbeads (MACS; Miltenyi Biotec, Auburn, California). Cells were incubated on ice with anti-F_cR mAb (2.4G2; American Type Culture Collection, Rockville, Maryland) to block F_c binding sites. Cells were stained with APC-labeled anti-CD11c (HL3), FITC-labeled anti-CD80 (16-10A1), and PE-labeled CD86 (GL1), all from Pharmingen. Live $CD11c^{hi}$ cells were gated for further analysis.

Peptide synthesis The K^b-restricted peptide OV8 (SIINFEKL) and the control K^b-restricted peptide 2C (SIYRYYGL) (Udaka *et al*, 1996) were prepared using Fmoc chemistry, purified by reverse phase high-performance liquid chromatography to $>80\%$ purity at the Nucleic Acid and Peptide Synthesis Facility of the University of British Columbia.

Statistical analysis Groups were compared using two-tailed Student's *t* tests or the Kruskal–Wallis test, with *post hoc* multiple comparisons where indicated. Results were analyzed and displayed using Prism 3 (GraphPad software, San Diego, California). The tumor survival curves were analyzed using SAS statistical software and the proc mixed procedure.

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