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Enhancement of recombinant adenovirus vaccine-induced primary but not secondary systemic and mucosal immune responses by all-*trans* retinoic acid

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Abstract

Vaccination is an important tool for enhancing immune responses against mucosal pathogens. Intramuscularly administered adenovirus (Ad) vectors have been demonstrated to be strong inducers of both systemic and mucosal immune responses. Further enhancement of immune responses following Ad vaccination is highly desirable. All-*trans* retinoic acid, a biologically active vitamin A metabolite, has been explored as an adjuvant for primary immune responses following vaccination. In this study, we investigated the effect of ATRA on a heterologous Ad prime boost regimen. All-*trans* retinoic acid (ATRA) co-administration during priming increased mucosal and systemic antibody responses as well as mucosal but not systemic CD8⁺T cell responses. However, this effect was no longer apparent after boosting regardless of whether ATRA was administered at the time of priming, at the time of boosting, or at both immunizations. Our findings confirm ATRA as an adjuvant for primary immune responses and suggest that the adjuvant effect does not extend to secondary immune responses.

Introduction

The incidence of sexually transmitted infectious diseases is increasing. Vaccines to sexually transmitted pathogens are thus far only available for some types of human papilloma virus and hepatitis B viruses. Vaccines to other pathogens such as HIV-1, herpes simplex virus type 2, *Treponema pallidum*, *Neisseria gonorrhoeae* or others that infect through the mucosa of the genital tract remain elusive. Correlates of protection against genital infections remain ill defined, but one would assume that prevention or limitation of infection would require immune effectors such as specific antibodies or CD8⁺ T cells at the port of the pathogen's entry.

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Lymphocyte homing patterns are dictated by the site of their induction mainly through imprinting by local dendritic cells (DCs) [1,2]. T and B cells expressing mucosal homing molecules such as CCR9 and $\alpha 4\beta 7$ are generally induced by mucosal immunizations [3,4,5], which target mucosal antigen presenting cells (APCs). They can also be stimulated by systemic immunizations in the presence of certain adjuvants that modulate DC functions [6,7]. CCR9 and $\alpha 4\beta 7$ expression on CD8⁺ T cells can be induced by antigen given together with all-*trans* retinoic acid (2E, 4E, 6E, 8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoic acid (ATRA) [8,9], which through a positive feedback loop induces retinoic acid (RA) synthesizing enzymes such as retinaldehyde dehydrogenase (RALDH), thereby increasing RA production. Previous studies demonstrated that ATRA given with antigen targeted to APCs in the skin such as by subcutaneous delivery induces gut-homing T cells and gut-homing IgA-producing plasma cells, which provide protection against pathogens that invade through mucosal surfaces [9].

We previously tested different routes of immunization with Ad vectors for induction of mucosal transgene product-specific B and T cell responses. Intranasal (i.n.) and oral immunizations induced strong genital IgA responses while intramuscular (i.m.) immunization of mice resulted mainly in IgG2a antibodies in blood and at mucosal sites [10]. Ad vectors given i.m. induced higher and more sustained frequencies of specific CD8⁺ T cells within the genital tract as well as in systemic compartments compared to i.n. immunization [11]. I.m. boosting with a heterologous Ad vector increased genital and systemic responses [11].

The present study was conducted to assess if ATRA given at the time of immunization with Ad vectors derived from chimpanzee serotypes (AdC) further increased genital homing of transgene product-specific immune responses, specifically CD8⁺ T cells and antibodies. In addition, we assessed whether ATRA modulated systemic responses, overall distribution of T cell subsets, or expression of CCR9 on different T cell subsets. Our results show that ATRA given at the time of priming markedly increases mucosal transgene product-specific CD8⁺ T cell responses without affecting systemic responses. ATRA administration in the context of a prime boost regimen had no apparent effect on responses measured after boosting. By the same token ATRA included in a single vector immunization regimen increased both systemic and genital transgene product-specific IgG but not IgA responses and was not effective as part of a prime boost regimen.

Results

Effect of ATRA on AdCgag vector-induced T cell responses

To test if treatment with ATRA modulates AdCgag vector-induced T cell responses, we injected female BALB/c mice i.m. with 10^{10} vp of an AdC6 vector expressing gag of HIV-1. Some of the mice were concomitantly given ATRA at 300 μ g in PBS intraperitoneally (i.p.). Mice were boosted 8 weeks later i.m. with an AdC7gag vector given at the same dose. For booster immunizations mice that had or had not received ATRA during priming were split into two groups; one received ATRA at the time of the boost, the other did not. Mice were bled periodically to analyze T cell subsets in blood (Figure 1). Different groups of mice were euthanized 8 weeks after priming and 8 weeks after the boost to conduct similar

analyses for T cells from spleens (Figure 2) and the genital tract (Figures 3,4). T cells from blood and spleens of individual mice were analyzed for numbers of CD4⁺ and CD8⁺ T cells as well as for memory subsets identified by expression of CD44 and for subsets with increased surface levels of CCR9. Gag-specific CD8⁺ T cells were identified by staining with an MHC class-I tetramer specific for the immunodominant epitope of Gag in H-2^d mice. Results were normalized to numbers of cells of a given subset within 10⁶ live lymphoid cells.

In blood, ATRA given once only affected marginal changes in numbers of circulating total CD4⁺ T cells. In the prime boost experiment, a single dose of ATRA caused a slight reduction in CD4⁺ T cells 16 weeks after the prime ($p = 0.014$). A slight reduction was also observed at 8 weeks after the boost in the two groups of mice that did not receive ATRA at the time of priming ($p = 0.011$). Numbers of CD4⁺CCR9⁺ cells, which comprised ~3% of the entire circulating CD4⁺ T cell population, were not changed by vaccination or ATRA treatment. Approximately 1/3 of all CD4⁺ T cells expressed CD44 indicating previous encounter with antigen and within CD4⁺CD44⁺ cells, 7.8% expressed CCR9; again these number were unaffected by inclusion of ATRA into the immunization regimen (Figure 1A-D).

ATRA did not change numbers of circulating CD8⁺ T cells, although their numbers were significantly increased after AdC6gag vector immunization compared to naïve animals (at 8 wks after priming $p = 0.0002$ for the no ATRA group, $p = 0.003$ for the ATRA group, at 8 weeks after the boost $p < 0.00001$ for all groups). CCR9 was expressed on approximately 50% of circulating CD8⁺ cells. At 8 weeks after AdC6gag vaccination ATRA caused a significant increase in numbers of CCR9⁺CD8⁺ T cells ($p = 0.028$) in blood. AdC6gag vaccination caused significant increases in circulating CD8⁺CD44⁺ T cells in most groups (no ATRA, 8 wks after prime $p = 0.003$, all other groups $p < 0.0001$), excluding the group that was tested 8 weeks after a single dose with AdC6gag given with ATRA. In naïve mice ~2% of CD8⁺CD44⁺ cells expressed CCR9, this percentage increased to ~7% in the vaccine groups, a difference that was significant after priming with ($p < 0.0001$) or without ATRA ($p = 0.002$) (Figure 1E-H).

AdC6gag immunization induced a robust gag-specific CD8⁺ T cell response, which by 2 weeks after immunization comprised ~2% of all circulating CD8⁺ T cells; frequencies and numbers declined over time. Gag-specific CD8⁺ T cell frequencies initially increased after the AdC7gag boost to 14% and then contracted over time. ATRA given during priming, boosting or both had no effect on numbers of Gag-specific CD8⁺ T cells in blood. Only a fraction of Gag-specific CD8⁺ T cells expressed CCR9; ATRA had no effect on numbers of Gag-specific⁺CD8⁺ CCR9⁺ T cells. Overall changes in circulating T cells following vaccination were largely driven by AdCgag vector vaccination rather than by ATRA (Figure 1I,J).

In spleens, ATRA also had only minor effects on CD4⁺ or CD8⁺ T cells. Increases in numbers of CD8⁺CD44⁺ T cells were seen in the prime boost experiment across all groups and most likely reflect an effect of either the repeated AdCgag vaccination or differences in age and an accumulation of antigen-experienced T cells during the additional 8 weeks that

were needed for the boost. Frequencies of Gag-specific CD8⁺ T cells were lower than in blood but again not affected by ATRA (Figure 2A-J).

We were able to isolate on average $1-2 \times 10^4$ lymphocytes from the genital tract of each mouse and therefore had to pool cells from 5 mice for each analysis. Experiments were conducted repeatedly and Figure 3 shows results for two representative experiments. As for blood and spleen, numbers were normalized to 10^6 live cells. Vaccine-induced changes in CD4⁺ T cells within the genital tract were subtle. Surprisingly, more than 50% of CD4⁺ T cells isolated from the genital tract lacked expression of CD44. In naïve mice ~ 4% of genital CD4⁺ T cells expressed CCR9; ATRA did not affect frequencies of CD4⁺CCR9⁺ T cells after priming. Upon priming, mice that received only the vaccine had higher numbers of CD4⁺CCR9⁺ or CD4⁺CD44⁻CCR9⁺ T cells compared to mice that received ATRA or compared to control mice (by one-way ANOVA with Bonferroni correction for multiple comparisons, adjusted p-values: $p = 0.031$ and 0.0096 , respectively). Differences were no longer significant after the boost. Numbers of CCR9⁺ cells within CD4⁺CD44⁺ T cells mirrored those of total CD4⁺ T cells, while numbers tended to be lower within CD4⁺CD44⁻ T cells (Figure 3).

Relative numbers of genital CD8⁺ T cells increased after priming or boosting although this did not reach significance. Increases were caused by vaccination rather than by ATRA. CCR9 expression was more common on genital CD8⁺ than CD4⁺ T cells. Vaccination given with or without ATRA had no major effect on numbers of CD8⁺CCR9⁺ cells. Significant differences were only seen for CD8⁺CD44⁺CCR9⁺ (adjusted p-value: 0.034); in this group priming with the vaccine increased relative numbers of these cells when compared to control animals (Figure 3).

At 2 weeks after AdC6gag priming ~4% of genital CD8⁺ T cells bound the Gag-specific tetramer. ATRA treatment increased their frequencies to 15%. As ATRA also increased relative numbers of CD8⁺ T cells, the differences in relative numbers of tetramer⁺CD8⁺ T cells within the genital tract was even more impressive (5.8 fold). Relative numbers of tetramer⁺CD8⁺ T cells within the genital tract increased by 4 weeks after the boost and again were markedly higher in ATRA treated mice. Inclusion of ATRA had no effect on relative numbers of Gag-specific CD8⁺ T cells at either 4 or 8 weeks after the boost nor did it affect T cell contraction (Figure 4). CCR9 was only detected at low numbers on tetramer⁺CD8⁺ T cells.

Effect of ATRA on AdCgp140 vector-induced antibody responses

To determine if ATRA modulates B cell responses, BALB/c mice were immunized as described above with vectors expressing gp140 of HIV-1 clade C (Du422). ATRA was given at the time of priming or boosting or at both time points. Mice were bled shortly before priming, 4 and 8 weeks after priming and 4 weeks after the boost. Vaginal lavage was harvested at the same time points. Antibody titers of samples from individual mice were measured by ELISA on plates coated with HIV gp140.

As shown in Figure 5, in mice that received ATRA, antibodies to gp140 developed by 2 weeks after priming. At this time, mice that only received the vaccine without ATRA had

barely detectable levels of gp140-specific antibodies. By 8 weeks after priming, gp140-specific antibodies increased in sera but not in vaginal lavage. Titers remained higher in mice that had received ATRA during priming. The AdC7gp140 boost markedly increased antibody titers in mice that had not received ATRA for priming but only had a marginal effect on antibody titers in mice that had been treated with ATRA during the prime. Adding ATRA to the boost had no effect on levels of gp140-specific antibodies in sera. In vaginal lavage, all groups showed enhanced gp140-specific antibody titers after the boost. Titers were comparable between the groups indicating that ATRA had no effect on B cell responses elicited by the boost. Samples were tested in addition for gp140-specific IgA responses; such responses could not be detected (not shown). Results of a 2nd experiment are shown in Supplemental Figure 1. Again in this experiment ATRA significantly increased systemic and mucosal antibody responses after priming but was ineffective after booster immunization.

Discussion

The majority of HIV-1 infections occur through mucosal transmission and in 70–80% of infections, a single virus or virus-infected cell establishes productive clinical infection [12]. This observation suggests the presence of natural immunological barriers to HIV-1 in the genital mucosa, which could potentially be enhanced by vaccination. We previously demonstrated that homologous and heterologous prime boost regimens consisting of intramuscularly administered Ad vectors elicited both systemic and mucosal antigen-specific CD8⁺ T cells [11]. Previous research by others showed that RA produced by DCs can act on both T and B cells and induce expression of CCR9, a mucosal homing receptor [1], and promotes class-switching to IgA in B cells [13]. In addition, ATRA (an isomer of RA) had been shown to increase both systemic and mucosal immune responses when co-administered with several vaccine candidates [8,14,15,16,17,18,19]. Ad vectors have been shown previously to induce the retinoic acid pathway upon transduction of DCs in vitro [20]. Nevertheless, this may not necessarily translate into their ability to promote mucosal homing of induced adaptive immune responses in vivo; Ad vectors only inefficiently infect DCs and stimulate transgene product-specific T cell responses primarily upon cross-priming [21], which would be expected to involve DCs that not matured due to direct contact with the vectors but rather as a results pro-inflammatory cytokines released by other infected cells. We therefore sought to determine if co-administration of ATRA and Ad vectors could increase mucosal immune responses to Ad vectors expressing transgene products that stimulate humoral or cellular immune responses. Specifically, we use Gag to test for effects on CD8⁺ T cells as Gag-specific CD8⁺ T cell responses have been associated with control of viral loads in human HIV-1 infected subjects as well as in preclinical vaccine trials [22,23]. Vectors expressing Env, the sole target for HIV-1 neutralizing antibodies, which have been shown in some trials to prevent retroviral acquisition [24], were used to test for an effect of ATRA on B cell responses.

Our data confirm that ATRA selectively increases recruitment of vaccine-induced Gag-specific CD8⁺ T cells to the genital tract without increasing systemic responses. Effects on overall systemic CD8⁺ and CD4⁺ T cells or recruitments of such T cells to the genital tract were subtle after priming or boosting. Importantly we did not observe increases in activated

CD4⁺ T cells within the genital tract which would have been be of concern for vaccines that aim to prevent genital transmission of HIV-1 as activated CD4⁺ T cells serve as viral targets [25,26] and their increase at the port of viral entry may facilitate transmission [27]. After booster immunization, ATRA given at the time of priming or boosting or both ceased to affect increases in Gag-specific CD8⁺ T cells within the genital tract. ATRA administration after priming or boosting failed to cause pronounced increases in CCR9 expression on CD4⁺ or CD8⁺ T cells, including Gag-specific CD8⁺ T cells, in blood or spleens as reported previously [8] or within the genital tract. This may reflect the timing of our analyses, which would not have detected transient changes during the T cells' early expansion phase.

Ad vectors given systemically induce transgene product-specific IgG responses while mucosal delivery elicits IgA responses [10]. ATRA has been reported to promote Ig switching to IgA even upon systemic application of antigen [9]. In our study, ATRA administration after priming caused a significant increase in systemic Env-specific IgG titers. Increases in genital Env-specific IgG titers were also observed upon priming although by peaking earlier they followed different kinetics compared to serum responses. ATRA failed to promote Env-specific IgA responses systemically or within genital secretions. As was observed for transgene product-specific T cells, ATRA failed to increase systemic or genital antibody responses in prime boost regimens.

Lack of an ATRA effect upon booster immunization was unexpected. Homing patterns of T cells are generally imprinted upon priming [1] and should have persisted after the boost. Lack of increased Gag-specific CD8⁺ T cells in mice primed in presence of ATRA suggest that ATRA may have imprinted mucosal homing preferentially onto terminally differentiated effector cells that failed to expand upon re-exposure to antigen. Lack of an ATRA effect given during the boost may reflect that ATRA selectively affects naïve rather than memory T cells.

ATRA administration caused a significant increase in Env-specific antibodies upon priming, which presumably reflected stimulation of increased numbers of ASCs. This was apparently not linked to increased induction of memory B cells. Mice that only received ATRA at the time of the boost fail to develop higher antibody titers, which again may reflect that ATRA primarily modulates responses of naïve B cells. Alternative explanations such as differential effects of ATRA on naïve versus memory follicular T helper cells, which are required for B cell maturation within germinal centers [28,29], also have to be considered.

In summary, our results show that ATRA serves as an adjuvant to increase genital immune responses after a single dose vaccine regimen. Its usefulness in more complex prime boost regimens could not be confirmed.

Materials and Methods

Construction of recombinant Ad vectors

Purified E1-deleted Ad vectors expressing Gag of HIV-1 clade B or gp140 of HIV-1 clade C derived from simian serotypes C6 (AdC6gag and AdC6gp140) or C7 (AdC7gag and AdC7gp140), were produced and quality controlled as described previously [11,30]. The

virus particle to infectious unit ratios of the four vectors used throughout the study were as follows: AdC7gag: 44.2, AdC6gag: 179, AdC7gp140: 493, AdC6 gp140: 400.

Animals and immunization

4–6 week old female BALB/c mice were purchased from the National Cancer Institute. Groups of 5 mice were immunized intramuscularly with 10^{10} virus particles (vp) of AdC6gag mixed with 10^{10} vp of AdC6gp140 diluted in PBS and administered into the tibialis anterior muscle of each hindlimb. For prime-boost experiments, mice were boosted i.m. with 10^{10} vp of AdC7gag mixed with AdC7gp140 8 weeks after the first immunization. Mice were housed at the Animal Facility of the Wistar Institute and all procedures used approved institutional protocols. Priming experiments were conducted 4 times in independent experiments; the prime-boost regimens were tested twice.

Preparation of samples

Serum was collected by submandibular bleeding and tested. For vaginal lavage, the vaginal cavity was rinsed three times with 60 μ l of sterile PBS. An equal volume of 0.01M dithiothreitol solution was added to each sample and left at room temperature for 1 hour with periodic vortexing. Debris was removed by centrifugation and samples were frozen at -20°C until assayed.

Isolation of lymphocytes

Peripheral blood mononuclear cells, spleens, and genital tracts were harvested as described [11].

Reagents

All-trans-retinoic acid (ATRA) (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at 40 mg/ml and stored as aliquots in the dark at -80°C . A working solution of ATRA (3 mg/ml) was prepared by dilution into PBS, followed by vortexing until ATRA dissolved. One dose of ATRA at 300 $\mu\text{g}/100 \mu\text{l}$ was delivered through i.p. injection. ATRA and vectors were administered concurrently.

Tetramer and lymphocyte marker staining

Tetramer staining was performed as previously described [11]. Briefly, lymphocytes were stained using allophycocyanin-conjugated major histocompatibility complex class I (H-2K^d) peptide (AMQMLKETI) tetramer (NIH tetramer core facility), AmCyan fluorescent reactive dye (Invitrogen), anti-CD8a-Pacific Blue, CD44-Alexa700, CD62L- PerCPCy5.5, CCR9-PE (eBioscience, San Diego, CA), CD4-FITC. Unless otherwise noted, antibodies were purchased from BD Biosciences. Prior to analysis, cells were fixed with BD Stabilizing Fixative (BD Bioscience). Flow cytometric analyses of lymphocytes were performed with a BD LSR II (Becton-Dickinson) flow cytometer. Data were analyzed using FlowJo V8.8 software (TreeStar). BD CompBeads Compensation Particles (Becton-Dickinson) were used to set distinct negative- and positive-stained populations. Gating schemes are shown in Supplemental Figure 2.

ELISA

Sera and vaginal washes of individual mice were tested for gp140-specific antibodies by ELISA on plates coated with a baculovirus-derived gp140 protein as described [31] or with a recombinant SIVgp160 (NIH AIDS Research and Reference Reagent Program). Briefly, 96-well plates were coated overnight at 4°C with HIV-1 gp140 (strain DU422) protein (150 ng/well). Wells were washed with PBS/0.05% Tween-20, followed by overnight blocking at 4°C with PBS/3% BSA/0.05% Tween-20. Wells were then washed and incubated with serial-diluted samples (in duplicates) for 2 hours at room temperature. Wells were subsequently washed, and bound IgG was detected with a goat anti-mouse IgG alkaline phosphatase conjugate (Sigma). Bound enzyme was detected with DEA substrate (KPL) and read on a microplate reader at 405 nm.

Statistical analysis

Experiments were conducted repeatedly using 5 mice per group. Results show the means $\pm$ SD. Significances between two groups were analyzed, by one-tailed Student's *t*-test. Three or more groups were compared by one-way Anova with Bonferroni correction for type 1 errors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. T Cell Responses in Blood

Mice were primed with AdC6gag vectors with or without ATRA. They were then split into 2 groups each and boosted with AdC7gag given with or without ATRA. PBMCs from individual mice were analyzed 8 weeks after priming and boosting. Naïve animals were analyzed in parallel. Graphs show numbers of cells of a given subset normalized to 10^6 live lymphoid cells as floating bars (min to max) with line at mean. Tet – Gag-specific MHC class I tetramer. * indicates significant differences to results obtained with animals that did not receive ATRA (by student t-test).

Figure 2. T cell Responses in Spleens

Mice were primed and boosted with or without ATRA as described in legend to Figure 1. Immunized and naïve mice were euthanized 4 weeks after the prime and 8 weeks after the boost and splenic T cells from individual mice were analyzed. Graphs show numbers of cells of a given subset normalized to 10^6 live lymphoid cells as floating bars (min to max) with line at mean. Tet – Gag-specific MHC class I tetramer. * indicates significant differences to results obtained with animals that did not receive ATRA (by student t-test).

Figure 3. CD4⁺ and CD8⁺ T Cells within the Genital Tract

Mice were primed and boosted with or without ATRA as described in legend to Figure 1. Immunized and naïve mice were euthanized 4 weeks after the prime and 8 weeks after the boost. Cells were isolated from the genital tract and pooled before staining. Graphs show numbers of cells of a given subset normalized to 10^6 live lymphoid cells as bar graphs. White bar graph – animals that received vaccines without ATRA. Light grey bar graph – animals that received ATRA during priming but not during the boost. Grey bar graph – mice that received ATRA only during the boost. Dark grey bar graph – animals that received ATRA both during priming and boosting. Naïve animals were analyzed at both time points, naïve animals analyzed at 4 weeks after priming are shown as white bar graphs, those that were analyzed at 8 weeks after the boost are shown as grey bar graphs.

Figure 4. Gag-specific CD8⁺ T Cells within the Genital Tract

This graph shows Gag-specific CD8⁺ T cells from the same groups analyzed at 4 weeks after priming, 4 and 8 weeks after the boost. Shading of bars is as described in Figure 3.

Figure 5. Systemic and Genital Env-Specific Antibody Responses

Mice were immunized as described in legend to Figure 1. Sera and genital wash were analyzed prior to vaccination (day 0), 4 and 8 weeks after priming and 4 weeks after the boost on plates coated with SIVgp140. Samples from animals that received vaccine without ATRA are shown as open squares, those from animals that received ATRA during priming but not upon boosting are shown as closed squares. Samples from animals that received ATRA only during the boost are shown as open circles, animals that received ATRA during both immunizations are shown as closed circles. * indicates significant differences to results obtained with animals that did not receive ATRA (by student t-test).