Computational modeling of the immune response to tumor antigens

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Abstract

Vaccination protocols designed to elicit anti-cancer immune responses have, many times, failed in producing tumor eradication and in prolonging patient survival. Usually in cancer vaccination, epitopes from one organism are included in the genome or linked with some protein of another in the hope that the immunogenic properties of the latter will boost an immune response to the former. However, recent results have demonstrated that injections of two different vectors encoding the same recombinant antigen generate high levels of specific immunity.

Systematic comparison of the efficacy of different vaccination protocols has been hampered by technical limitations, and clear evidence that the use of multiple vectors has advantages over single carrier injections is lacking. We used a computational model to investigate the dynamics of the immune response to different anti-cancer vaccines based on randomly generated antigen/carrier compounds. The computer model was adapted for simulations to this new area in immunology research and carefully validated to the purpose. As a matter of fact, it reproduces a relevant number of experimental observations.

The model shows that when priming and boosting with the same construct, competition rather than cooperation develops amongst T cell clones of different specificities. Moreover, from the simulations, it appears that the sequential use of multiple carriers may generate more robust anti-tumor immune responses and may lead to effective tumor eradication in a higher percentage of cases. Our results provide a rational background for the design of novel strategies for the achievement of immune control of cancer.

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1. Introduction

Anti-cancer vaccination is based on the existence of antigens, selectively or preferentially expressed by tumors, called tumor-associated antigens (TAAs).

Cancer eradication using TAA vaccination has been demonstrated in numerous animal models. However, active immunotherapy of cancer in human beings has achieved very limited success to date (Finn, 2003). The compromised immune system of patients and the high tumor burden have been blamed as largely responsible for the clinical failures (Finn, 2003; Minev, 2002; Lisoc et al., 2001; Nouri-Shirazi et al., 2000; Ribas et al., 2003; Stevenson et al., 2004). Furthermore, in order to induce a stronger anti-tumor response, a number of challenges need to be faced such as what antigens to use, what schedule of vaccination to employ and what adjuvants to add to the inoculum. In fact, tumor antigens are known to be weak immunogens that hardly trigger an effective immune response.
Most clinical trials contemplate multiple injections of one TAA (mainly in the form of protein, recombinant DNA or viruses modified to express TAA) inoculated along with one highly immunogenic molecule (named carrier). Carriers are typically derived from organisms that are phylogenetically very distant from the host (the genome of recombinant microorganisms engineered to express TAA can be regarded as a carrier itself).

However, recent results have demonstrated that injections of two different vectors encoding the same recombinant antigen (e.g. priming with plasmid DNA and boosting with recombinant modified vaccinia Ankara) generate high levels of specific immunity (Amara et al., 2001; Schneider et al., 1998).

Of the many thousands of peptides encoded by a complex antigen potentially presented to CD8T cells, only a small fraction induces a non-negligible response in association with any given MHC class I allele, a phenomenon known as immunodominance (Yewdell and Bennink, 1999). This issue should be considered in order to design vaccination strategies able to induce an optimal cytotoxic T cell mediated immune response.

In order to evaluate the effects on both humoral and cellular immune response of repeated injections of TAA together with carriers, we used computer simulations based on a modified version of a well established computational model (Celada and Seiden, 1992). In particular, we compared protocols by using multiple different vectors/carriers and correlated the frequency of anti-tumor T cells and antibody titers with tumor control.

2. Modeling the immune response to cancer

The microscopic model we employ represents the most important entities of the immune system at cellular level and basic immunological processes including hematopoiesis, thymus selection of CD4 and CD8 T lymphocytes, antigen digestion and presentation (endogenous and exogenous pathways) by antigen processing cells (B lymphocytes, macrophages, dendritic cells), hypermutation of antibodies and cytotoxicity by CD8 T cytotoxic lymphocytes (Bernaschi and Castiglione, 2001; Bernaschi and Castiglione, 2002; Castiglione et al., 2003; Celada and Seiden, 1992; Celada and Seiden, 1998).

The model is polyclonal, meaning that different clones of lymphocytes are represented at the same time. In particular, each lymphocyte is equipped with a cell receptor drawn from a suitable mathematical space of representation. Here we use the space of binary strings. Hence, with a binary string of length \( l \), we represent a repertoire of size \( 2^l \). Although much smaller than the realistic estimated repertoire of B cells or T cells (about \( 2^{56} \) for B cell receptors and \( 2^{53} \) for T cell receptors (Perelson and Weisbuch, 1997), \( l = 12 \) gives a repertoire which is sufficient for our purpose. Therefore, cell receptors, but also antigens (hence the tumor peptide and the carrier) and immunoglobulins, are represented in the discrete space \( \{0,1\}^{12} \). Actually we use two binary strings for the epitopes and two binary strings for the peptides; therefore an antigen is an object of the space \( \{0,1\}^{48} \).

Immunogenicity of a molecule is defined as the Hamming distance (that is the number of complementary bits in the bit-wise comparison) from the subset representing the repertoire of the self-molecules. For the sake of simplicity, the self is defined by a single molecule and we set the TAA equal to the self-molecule. Therefore, any other molecule belongs to the non-self (i.e. the carriers are randomly chosen in \( \{0,1\}^{12} \) but not equal to the TAA).

The interactions among entities are depicted in Fig. 1 where dashed lines indicate “non-specific” bindings whereas solid lines indicate “specific” bindings. Here the term “specific” means that the binding is modeled through a random boolean variable whose probability is an exponential function of the Hamming distance between the binary strings involved in the molecular recognition. In contrast, non-specific binding is equivalent to have a binding rate equal to a certain constant.

Interactions in Fig. 1 can be summarized as follows: antigens are captured and processed by antigen presenting cells (dendritic and B cells). Dendritic Cells (DCs) activate cytotoxic CD8T cells (TCs) upon recognition of the TCR with the MHCI-peptide molecule. Activated TCs are stimulated to proliferate. Moreover, activated TCs can kill MHCI-peptide bearing cells (i.e., DC as well as Cancer Cells, CCs). Stimulated TCs can enter the duplication phase but only in presence of IL-2 which is produced by CD4T cells (Th cells). In turn, T helper cells produce IL-2 upon stimulation by APCs represented by macrophages (MA), B lymphocytes (B) and dendritic cells (DC).

A time step of the simulation corresponds to 8h of real life. Cells and molecules interact in a simulated space that corresponds to multiples of one milliliter of blood. However, given the time resolution, cells are allowed to circulate to lymph nodes where the presentation of the antigen by APCs takes place. The concept of physical proximity is enforced so that cells reside on a two-dimensional lattice and interactions may happen only among cells and molecules living in the same lattice-site (Fig. 1 panel (b)).

The simulation is carried out by iterating the following steps:

Step 1: Generation of new cells from the bone marrow and thymus selection of immature CD4/CD8 T lymphocytes.

Step 2: Interaction among entities executed in random order to avoid any bias toward a particular interaction.
This step includes all interactions depicted in Fig. 1 panel (a).

Step 3: Random diffusion of cells and molecules. However, at this stage, we do not take into account any real estimate of the cell mobility coefficient (Miller et al., 2002). To prevent unrealistic accumulations of cells in one lattice site, diffusion is constrained by the cell density of the lattice site of destination. To this purpose, we assume that the whole lattice represents 10⁹ m³ and that all cells are perfect spheres with an average diameter of 10 μm. Under these assumptions, the probability to diffuse to a neighboring lattice site chosen at random is set equal to 1 minus the ratio between the current number of cells occupying the destination site and the maximum number of cells that can occupy one lattice site (about 8000 in a 16 x 16 lattice).

Step 4: Duplication of stimulated cells. Clone division is performed at a rate of one division each time step and five divisions per stimulated cell (since a time step is equivalent to 6–8 h and a stimulated cell duplicates for about one and half day). During division, cells cannot interact with other entities. Division of T cells is constrained by the availability of IL-2 in the same lattice site. This requirement, together with the very short life-time of the IL-2, mimics the paracrine and autocrine nature of the action of cytokines.

Without an antigenic stimulus, the population of cells is at equilibrium, that is, the turnover is constant. Deviations from the equilibrium are triggered by the presence of antigens. The model has been used to simulate both bacterial and viral infections (Bernaschi and Castiglione, 2002; Celada and Seiden, 1992, 1998), but this is the first time we use it to simulate the immune reaction against a tumor. To do so, we set a tumor mass (i.e. an aggregation of a relatively large number of cells) in a background of healthy immune cells. Since cancer cells expose tumor fragments on the MHC I molecules, they should be recognized by the CD8 T cells and destroyed. However, as in reality, this does not happen because of the poor immunogenity of the TAAs. In our model this is represented by the fact that a very small fraction of autoreactive T cells passes the thymus selection phase. As a result, without explicit vaccination, the clone of autoreactive T cells, if present, is not able to mount an effective immune response to clear the tumor.

In the beginning of the simulation, all cancer cells are in the center of the lattice. From this viewpoint it can be considered as a solid tumor.

In a number of studies it has been found that growth of tumor cells population is exponential when the number of tumor cells is small whereas growth is slower when the population size increases (Dhodapkar et al., 1993). For this reason the cancer cell population doubles in a (simulated) year. According to biological features of so-called “chronic” or “indolent” malignancies, this is the scenario of choice in the clinical setting of active immunotherapy.

Methods of active immunotherapy employed in clinical practice have been previously reviewed (Finn, 2003; Liso et al., 2001; Minev, 2002; Ribas et al., 2003). We model cancer vaccines by administering the TAA alone or in conjunction with different carriers. Either case produces a different immune response that is able, or unable, to eradicate the tumor.

The microdynamics of the model can be summarized as follows: once injected into the host, the antigen
presenting cells capture the injected molecules composed by the low immunogenic TAA and the high immunogenic carrier epitopes, process them and then present the peptides on the MHCI molecules for the recognition by TC cells. At the same time, the APCs digest and present the peptides bound to the MHCI molecule that is then in charge for the presentation to the CD4T cell receptors. THs that bind the complex are stimulated and release IL-2. IL-2 sustains the proliferation of active CD8 cells. By injecting the TAA, one forces the stimulation of TAA-specific CD8T cells. Such cells recognize the MHCI-peptide molecules on the cell surface. This may happen on APCs but also on cancer cells. Upon recognition of the MHCI-peptide molecule, CD8T cells kill the target cells and enter the mitotic cycle.

2.1. The selection of the parameters

The parameters of the model are given in Table 1. They can be classified as (i) “well defined” parameters such that there is a vast consensus among immunologists about their value. Examples of this class of parameters are the initial number of lymphocytes and other cells per μl of blood (Goldsby et al., 2000) or the half-life of cells; (ii) parameters that define the problem under investigation (i.e. the initial conditions). Examples of this class of parameters are the simulated time horizon (about fifteen months), the simulation space (1 μl of blood), the initial tumor burden (10^3 cancer cells in a μl of blood or, equivalently, in a mm^3 of tissue) and the vaccine dose (this is 0.5 μg/ml, i.e. the resulting concentration is 0.5 μg in a μl of blood after a suitable amount of time); (iii) parameters whose value must be tuned by means of a series of preliminary tests. Note that, since the model has been used in a number of other studies (Bernaschi and Castiglione, 2002; Castiglione et al., 2003; Celada and Seiden, 1992), many of the parameters belonging to the third class have been already studied (for instance the hypermutation rate of antibodies Celada and Seiden, 1996). As to the other parameters in this class, we set the bit-string length l equal to 12 corresponding to a potential repertoire of 2^12 = 4096 distinct cell receptors, the DC affinity to antigens six times greater than in the MA case. The fraction of circulating autoreactive (anti-tumor) TH and TC cells is below 0.1% but not less than 0.01%. The size of the lattice is automatically adjusted in such a way that the system shows a credible primary humoral immune response to the injection of a generic antigen that peaks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Meaning</th>
<th>Value/range</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>Ab secretion rate</td>
<td>10 ng/ml/8 h</td>
</tr>
<tr>
<td>ν</td>
<td>Lymphocytes duplication steps</td>
<td>5</td>
</tr>
<tr>
<td>τ_B</td>
<td>Half-life of B lymphocytes</td>
<td>3 days</td>
</tr>
<tr>
<td>τ_TH</td>
<td>Half-life of TH lymphocytes</td>
<td>3 days</td>
</tr>
<tr>
<td>τ_TC</td>
<td>Half-life of TC lymphocytes</td>
<td>3 days</td>
</tr>
<tr>
<td>τ_DC</td>
<td>Half-life of dendritic cells</td>
<td>3 days</td>
</tr>
<tr>
<td>τ_MA</td>
<td>Half-life of macrophages</td>
<td>3 days</td>
</tr>
<tr>
<td>τ_Ag</td>
<td>Half-life of the injected antigenic compound</td>
<td>1 week</td>
</tr>
<tr>
<td>τ_IL2</td>
<td>Half-life of IL-2</td>
<td>&lt;1 day</td>
</tr>
<tr>
<td>τ_AB</td>
<td>Half-life of immunoglobulins</td>
<td>23 days</td>
</tr>
<tr>
<td>B(0)</td>
<td>B’s initial population</td>
<td>260 cells/mm^3</td>
</tr>
<tr>
<td>TH(0)</td>
<td>TH’s initial population</td>
<td>876 cells/mm^3</td>
</tr>
<tr>
<td>MA(0)</td>
<td>MA’s initial population</td>
<td>351 cells/mm^3</td>
</tr>
<tr>
<td>TC(0)</td>
<td>TC’s initial population</td>
<td>434 cells/mm^3</td>
</tr>
<tr>
<td>DC(0)</td>
<td>DC’s initial population</td>
<td>351 cells/mm^3</td>
</tr>
<tr>
<td>Parameter</td>
<td>Meaning</td>
<td>Value/units</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>φ</td>
<td>Vaccine dose</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>P_MAAg</td>
<td>Binding probability of MA to antigen</td>
<td>0.05</td>
</tr>
<tr>
<td>P_DC.Ag</td>
<td>Binding probability of DC to antigen</td>
<td>0.30</td>
</tr>
<tr>
<td>Σ</td>
<td>Maximal number of times a lymphocyte enters the mitotic cycle</td>
<td>5</td>
</tr>
<tr>
<td>η</td>
<td>Initial fraction of autoreactive T lymphocytes</td>
<td>0.01–0.1%</td>
</tr>
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in (about) 8–10 days. Finally, the secretion rate of antibodies by plasma cells in terms of the resulting concentration is set equal to 10 ng/ml each 8 h based upon in vitro data (monoclonal antibodies production, Goding, 1983).

These values fulfill the fundamental requirement that the resulting humoral and cellular response (in terms of certain observables) falls within an acceptable range of values by running the special cases described in the section “Model validation”. This is obviously only a qualitative measure that we obtain by monitoring curves like those in Fig. 2. For instance, if the value of the MA or DC’s affinity to the antigen is too small, we do not observe an immune response because we block antigen presentation; if the fraction of autoreactive lymphocytes is too small there is neither humoral nor cellular immune response. Finally, the choice of the bit-strings representing the MHC molecules and the carriers aims to achieve a significant, from a statistical viewpoint, sample of all the possibilities; i.e. running a sufficiently large number of simulations, we can compute average values of the observables as described in the “Results” section.

In the beginning of this section we mentioned that the half-life of the cells is a well-known parameter. Actually, there is an important exception that is the half-life of memory cells. The question of how long a memory cell lives is very controversial and the fact itself that some cells maintain memory of a previous infection remains to be proven. At this time, we implicitly define memory cells by means of a mechanism that automatically (and dynamically) changes their half-life after a successful binding. In other words, the stimulation of lymphocytes actively involved in the immune response is interpreted as a signal of their importance for the immune system defense and their half-life is increased as a consequence. This is quite reasonable if we neglect phenomena like anergy. The result of this process is that memory is formed by those cells that participate more actively in the defense of the organism during the immune response.

2.2. Model validation

The model has been validated in different situations. A general description of the cells behavior once stimulated by an anti-cancer vaccination is reported in the following paragraphs.

2.2.1. No treatment

We started simulating the very simple situation in which there is no treatment. In this case, the cancer cells population grows freely and there are few chances for the immune system to mount a cellular or humoral specific response against malignant cells because of the very scarce immunogenicity of TAA.

2.2.2. TAA alone

In case of injections of TAA alone, we observe, in accordance with a large body of experimental evidences (Finn, 2003) a very weak response. In particular, a set of 50 simulations (using a TAA dose equal to all other experiments) always ended with uncontrolled tumor growth, regardless of the vaccination schedule. In this scenario, since T cells undergo the selection phase in the thymus, very few autoreactive CD4T cells are able to stimulate the B lymphocytes maturation into antibody-secreting plasma cells. Moreover, since IL-2 is released by the active Th cells and is necessary for cytotoxic clone expansion, a very modest cytotoxic activity against the tumor is produced. As a result, the immune system is unable to compete with the tumor growth.

2.2.3. TAA+SINGLE carrier

In the third scenario the anti-cancer vaccination is realized by a combination of a TAA and a carrier (see Fig. 2). In this experiment, we administered ten times the same TAA + carrier compound with a carrier chosen at random (no special affinity to the MHC I molecule is assumed but a large immunogenicity, i.e. a greater distance from the self). Here, as in the experiments described above, the simulated space is equivalent to 1 ml of blood and the time span of the experiment is about half a year. Like in other studies conducted with this model (Bernaschi and Castiglione, 2002; Castiglione et al., 2003), realistic parameters for the concentration of lymphocytes as well as the half-life coefficients of cells and molecules are used.

In the simulation of injections of TAA plus a carrier with no particular affinity to the MHC I or the MHC II, we observed a greater cytotoxic response than in the two previous test cases. However, the response we observed was directed against the carrier more than against the TAA and corresponds to the experimental observations of immunodominance (Etlinger et al., 1990). The use of the same combination of TAA and carrier induces also
the immune system to mount a strong humoral response directed toward the carrier.

2.2.4. TAA+MULTIPLE carriers

Finally, the last scenario is given by the simulation of different carrier molecules at each administration with the same TAA. Briefly, this vaccination protocol enforces the immune system to “partially” start a new recognition process and a consequent primary response against the carrier at each injection. As a result, there is a large number of active Th cells secreting IL-2 and, at the same time, a lower number of competing antibodies.

2.3. Simulations

We performed a large set of simulations to evaluate the effects of administering the TAA alone or in combination with carrier molecules according to five different schedules of injection. The goal was to find how to induce a more robust anti-tumor immune response able to generate the largest number of TAA specific CD8T cells and leading to tumor eradication.

We performed two different sets of simulations with the same set of parameters. We called “No Rotation” (NR) the set of simulations in which all injections consist of the TAA combined with the same carrier whereas we called “Yes Rotation” (YR) the group where the carrier is changed randomly at each new injection.

In both experimental groups (NR and YR), ten injections were simulated at 1, 7, 14, 28 and 42 days interval respectively, resulting in ten sets of computer runs. For each group of experiments and for each vaccination protocol we repeated the numerical simulation 300 times starting from a different random initial condition to increase the reliability of the performed tests. This accounts for $300 \times 5 \times 2 = 3000$ simulations that required more than 100 h of computation on a workstation with two processors.

3. Results

3.1. Immune control of tumor

In the NR case, as we performed a set of independent runs by changing the random seed of the random number generator, we simulated a different carrier at each run (i.e. for each virtual patient). In the second case (Yes Rotation), it is the whole sequence of carriers chosen for the schedule which is different from run to run.

Fig. 3 shows the percentage of success (i.e. virtual survival) for the two situations (No Rotation vs. Yes Rotation) and for all injection schedules analysed. Any case in which the number of cancer cells at the end of the simulated time period (i.e. 430 days), is smaller than the number at the beginning of the therapy (i.e. 1000 cells/mm$^3$) is considered as a success.

Overall, it appears that carrier rotation (i.e. the YR scenario) is advantageous with respect to the situation in which the carrier is fixed. Moreover, the observed difference between the non-rotating and rotating carriers groups is larger for an interval between injections equal to 2 weeks and is minimal or null at the two extremes (i.e. for intervals of 24 h and 6 weeks) ($\chi^2$ tests indicate that for the 1, 7 and 14 days sets of simulations, the difference between the No- and Yes Rotation is significant whereas for the 28 and 42 days sets of simulation, it is not). For the vaccination simulations based on a rotating carrier, the 14 day-interval vaccination schedule is optimal, meaning that it triggers a more robust immune response and achieves higher tumor eradication rates.

We hypothesize that the 14-day interval is the best tradeoff between two opposite requirements: (a) higher frequency of injection that eases an early generation of the immune response, when the tumor mass is smaller and (b) enough time between injections for the development of anti-TAA memory. Finally, rotation prevents the immune response from focusing on one particular carrier.

3.2. Cytotoxic immune response

Notably, the 14-day interval induces the highest average number of TAA-specific cytotoxic T cells.
amongst all simulations (Fig. 4) and, at the same time, leads to the survival of the host (Fig. 3). Although delaying injections can induce a high number of anti-TAA CD8T cells in the long run (compared to the 1 day interval, but still smaller compared to the 7 and 14 days interval, see Fig. 4), it is obviously less effective in eradicating the tumor (Fig. 3). In other words, the development of a relatively strong immune response in the late stage when the tumor burden is exceedingly high is almost useless from the clinical viewpoint.

We can also conclude that a greater anti-carrier cytotoxic response is generated in the single carrier simulation since the total counts of CD8T cells in the system are comparable in all different schedules of vaccinations (data not shown), and the only two antigens in the simulations are the carrier(s) and the TAA, i.e. CD8T cells generated are directed against either TAA or the carrier(s).

The number of TAA-specific CD8T cells shown in Fig. 4 is estimated by taking a snapshot 3 months after the end of the therapy and only for those simulations in which tumor eradication was observed entered the calculation.

### Table 2

<table>
<thead>
<tr>
<th>Number of days between injections in the different vaccination protocols</th>
<th>Simulated time frame (days) at three months after the end of the therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td>153</td>
</tr>
<tr>
<td>14</td>
<td>216</td>
</tr>
<tr>
<td>28</td>
<td>342</td>
</tr>
<tr>
<td>42</td>
<td>430</td>
</tr>
</tbody>
</table>

*It should be day 468 but the simulation ends at day 430.

which totalized a larger titer of antibodies in the YR case (Fig. 5 upper panel).

Notably, by looking at the specificity of the produced antibodies (affinity to the TAA or to the carrier was assessed by counting the number of antibody-producing plasma cells), we found that a significant part of the humoral response with low affinity to TAA (conversely, high affinity to the carrier) is generated mostly in the NR protocols (Fig. 5 lower panel). In fact, the development of antibodies that do not react with sufficient affinity (i.e. below the threshold of 9 bits over 12) with the TAA reflects the generation of anti-carrier antibodies, since carriers are the only other antigen in the picture able to induce an immune response. Therefore, we conclude that in the NR experiments generation of significantly higher titres of anti-carrier antibodies is likely to be responsible, at least in part, for the lower anti-tumor effect observed in these groups.

Note that the hole in the cell population with match 6 and 7 in the lower panel of Fig. 5, is due to the “complementary nature” of the bit-string match.

### 3.3. Humoral immune responses

Fig. 5 shows the humoral immune response produced in the YR and NR scenarios. We report the total antibody concentration regardless of the affinity to the TAA and to the carrier. We found that titres of antibodies are slightly higher (the difference is in the order of 5–20%) in the NR groups for each schedule of vaccination with the exception of the 1-day-protocol

### 4. Discussion and conclusions

Vaccinology is often regarded as practical. Vaccines schedules are mainly empirical and based on the assumption that (sooner or later) an immune response can be generated and boosted by repetitive injections of antigens at times arbitrarily chosen.

The idea that the conjugation of a weak (tumor) antigen to a strong immunogen (carrier) is able to induce an anti-tumor immune response is probably oversimplifying and the development of a strong anti-carrier immune response may be harmful instead of beneficial.

Indeed, we have shown, by means of our computational model, how immune response to one given carrier does not necessarily favor immune response to weak antigens and after repetitive booster injections, anti-carrier can dominate over anti-tumor immune response. Moreover, we have shown that injections at a 14 day-
Fig. 5. Upper panel: titres of antibodies produced are higher in the No Rotation groups per each schedule of vaccination. Lower panel: Antibody-producing plasma cells with low affinity to TAA (anti-carrier) are generated mostly in the No Rotation protocols. The hole in the cell population with match around 6–7 is due to the “complementary nature” of the bit-string match.
interval induce the highest average number of TAA-specific cytotoxic T cells (Fig. 4) and the best survival rate (Fig. 3) when combined with multiple carriers.

Intriguingly, the difference in the results obtained with multiple carriers versus a single carrier is hampered when injections are delayed in time. Probably the capability of vaccination to control the increased tumor mass is reduced, in this case, by the increased time delay before the immune response is mounted.

As a matter of fact, the complex relationship between carrier molecules and non-immunogenic peptides (haptens) began to be elucidated when, with great surprise of most immunologists, carrier pre-immunization was shown to generally induce epitope suppression rather than help in host challenged with carrier-hapten conjugate (Herzenberg and Tokuhisa, 1980). The phenomenon was shown to be dependent on B cells specifically recognizing the carrier molecules (Leclerc et al., 1990).

Similarly, with regards to T cells, serotype-defined viruses that share most (if not all) T-helper and cytotoxic T-cell epitopes, do not induce strong cellular immune responses against each other. Actually, the opposite is true: the immune response to newly generated epitopes in the presence of strong (pre-existing) immunity is either strictly of primary kinetics (Liang et al., 1994) or even ineffective when immune system concentrates on dominant epitopes previously seen (Kleneman and Zinkernagel, 1998).

The explanation is that competition plays a pivotal role in shaping adaptive immune responses. Since resources in our body are limited and the number of antigens is enormous, this competition is instrumental to prevent redundant immune responses. In particular, competition is generated amongst circulating antibodies and dendritic cells to capture the injected TAA molecules; moreover competition develops almost invariably amongst different T cell clones. In particular, T cell competition has been demonstrated to drive affinity maturation (Kedl et al., 2003) and thus allows only the most suitable clones to emerge by enabling the system to focus on the most attractive epitopes.

T cell epitopes provided by carriers probably help (in generating anti-TAA antibodies and T cells) only if all the following conditions apply (Zinkernagel, 2002): (i) frequencies of relevant specificities exist (unlikely for TAA), (ii) T cell help is limiting for the developing immune response and (iii) competition for antigen presentation on a common APC does not develop.

Competition of T cells for space and growth factors has been suggested as a general mechanism (Bartholomew et al., 2003; Kedl et al., 2002; Stockinger et al., 2001). However, it remains unclear how all these factors are, in turn, regulated (Kedl et al., 2003; Smith et al., 2000). In our model, competition amongst T cell clones for APC is caused by space limitation (mass effect) and by the killing of DCs by CD8 Ts.

Notably, experimental evidence shows that improvement of anti-TAA immunity has been obtained through removal of competing carrier epitopes (Rice et al., 2002). Also, competition among CTLs has been demonstrated to limit the immune response (Palmowski et al., 2002), at least in specific experimental settings.

Indeed, our model also suggests that circulating antibodies and dendritic cells compete to capture the injected molecules. This is in accordance with the finding that natural antibodies switch immune response from cellular to humoral (Apostolopoulos et al., 1998). This is also confirmed by the sporadic evidence of immunization using recombinant adenoviruses (Rosenberg et al., 1998) due in part to pre-existing or rapidly generated circulating antibodies directed against envelope proteins on these viruses.

Bound antibodies in the real world can modulate antigen processing but it is not entirely clear to what extent this affects antigen presentation and if bound antibodies modulate T cell response towards or away from particular determinants (Smitil et al., 1995). However, it should be emphasized that current limitations of our model allow the inclusion of immune complexes only as immuno-inactive species.

Finally, although it has been shown that immune response can be generated in the presence of preexisting anti-carrier immunity (Rice et al., 2002; Timmerman et al., 2001), there is no evidence that this is the optimal strategy to pursue. Furthermore, animal models allow injection of much higher quantities of TAA per unit of body mass than the human trials, and an elevated number of antigens is likely to be needed to overcome the anti-carrier antibodies mediated effect.

In conclusion, although injection of the TAA alone appears to be insufficient to elicit an immune response against TAA-bearing cells because of the lack of co-stimulus and because of the limited number of naturally occurring autoreactive cytotoxic cells, injection of TAA plus a single carrier is probably not advisable. Vaccination strategies contemplating the use of multiple carriers deserve further testing both in animal models and in the clinical setting.

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