Recombinant vaccinia viruses expressing immunoglobulin variable regions efficiently and selectively protect mice against tumoral B-cell growth

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The variable regions of the immunoglobulin (Ig) expressed on the surface of a malignant B cell can be considered tumor-specific antigens and, as such, could be targets for immunotherapeutic approaches. However, because until now the immunization procedures have been complex and have given only partial protection, it was necessary to find new methods of immunotherapy. Here, we present a successful method of vaccination against B-cell tumors in a murine model. We produced recombinant vaccinia viruses (rVV) expressing the heavy and the light chain of surface Ig of a patient's malignant B cells and we tested the ability of these rVV to protect immunized mice against tumor growth of transfectomas producing the same human Ig. The protection of the mice was complete and specific to the variable region of the immunizing heavy chain although specific lymphoproliferative and cytotoxic responses were not detectable *in vitro*. The protection was strictly dependent on the presence of CD4 T cells and asialo GM1+ cells. Furthermore, tumor protection clearly required γ -interferon and was partially inhibited by blocking the Fas-Fas ligand interaction. We also show, in a murine syngeneic model, that rVV expressing a poorly mutated Ig protects against the growth of Ig-producing tumor. **Cancer Gene Therapy (2001) 8, 815–826**

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Human B-cell malignancies constitute about 75% of the cases of lymphoproliferative diseases in adults and include B-cell chronic lymphocytic leukemia (B-CLL), hairy cell leukemia, diverse non-Hodgkin's lymphomas, and multiple myeloma.¹ These B-cell malignant tumors are the result of single clone proliferation of cells expressing surface markers, of which the surface immunoglobulin (sIg) is the most specific: in fact, the genetic processes, which occur during B-cell ontogeny, give rise to an extremely diverse Ig repertoire, with each Ig being considered a tumor-specific antigen in B-cell malignancies.

Both active and passive approaches to immunotherapy in B lymphoproliferative diseases are of interest. The goal of active immunotherapy is to induce or to stimulate the cellular and/or the humoral anti-idiotypic, antitumor immunity. It gave promising results in murine models and clinical trials using tumor-originating Ig,^{2–7} and more recently using Ig processed by dendritic cells^{8,9} or DNA coding for the Ig in question.^{10–12} For instance, King et al induced protective immunity against murine lymphoma and myeloma using DNA vaccines containing tumor-derived single-chain Fv fused with the gene encoding the fragment C of tetanus

toxin. What could be the theoretical limitations of active immunotherapy? In the first place, it may be conjectured that self-antigens induce tolerance by causing the functional silencing or the physical elimination of high-avidity T cells. If this conjecture is the case, it could be of particular importance for nonmutated antigens, which constitute very weak tumor antigens. In the cases of B-cell malignancies, it will probably be more difficult to induce an immune response against germline variable region products (like some B-CLL^{13–15}) than against mutated forms (like follicular lymphomas^{16,17}). In the second place, the vaccination protocols were shown to be able to induce anti-idiotypic responses in the host, but were possibly not entirely satisfying in activating low-avidity T cells or other effector cells. An interesting alternative approach consists of the use of a recombinant vaccinia virus (rVV), which is known to offer several advantages as an immunization vector: (a) it can accommodate up to 26 kbp of foreign DNA, without loss of infectivity; (b) it is easier and less time-consuming to construct and produce than to purify large amounts of tumor-associated protein; (c) it can infect a large variety of cell lines; and (d) it is highly efficient in inducing long-lasting humoral and cell-mediated immunity and could therefore avoid the necessity of using other immune stimuli.^{18–21}

In order to test the ability of the vaccinia virus (VV) approach, we developed a two-step strategy: in the first step, we cloned the variable region genes of an Ig originating from

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the leukemic B cells of a single patient ARN suffering from B-CLL.²² The rearranged genes coding for the light (L) and heavy chains (H) were then cloned into VV expression cassette vectors. The rVV were evaluated for their capacity to protect mice against the tumor growth of transfectomas producing the same human Ig. In the second step, we studied the ability of this vaccinia approach to circumvent the possible immune tolerance against poorly mutated variable regions of Ig by using a syngeneic murine model.^{23,24}

MATERIALS AND METHODS

Recombinant vaccinia viruses

rVV expressing the human Ig genes. The preparation of the leukemic B cells of the patient ARN, the amplification, and the sequencing of the rearranged genes coding for the H and the L chains expressed by these cells have been described elsewhere.²² For the construction of the VV expression vectors, total RNA were isolated from the peripheral blood lymphocytes of the patient ARN, extracted with phenol/chloroform, and precipitated with ethanol. cDNA were then synthesized using reverse transcriptase (Boehringer Mannheim Biochemicals, Mannheim, Germany) and primers complementary to the 3' end of the C κ constant region and of the C μ 2 exon. The heavy-chain cDNA was subjected to polymerase chain reaction amplification using Taq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA) and primers specific to the VHII leader region and the 3' end of the C μ 2 constant region. The L chain cDNA was amplified using primers specific to the V κ III leader region and the 3' end of the C κ constant region. The H chain polymerase chain reaction product was cloned in the plasmid pTG186poly²⁵ upstream of the sequence coding for the intracytoplasmic and transmembrane domains of rabies glycoprotein,²⁶ giving pVVHt (Fig 1A). The V κ J κ C κ gene complex was cloned in the plasmid pTG186poly giving the pVVL vector (Fig 1B). Sequencing was carried out using primers specific to both the 3' and 5' ends to check that unwanted mutations were not introduced into the human sequences during the different procedures. The rVV were generated by *in vitro* homologous recombination of VV DNA (Copenhagen strain) with the plasmids pVVL, pVVHt, and pTG186poly in primary chicken fibroblasts, as described.²⁷ This resulted in the generation of the VVL, which synthesizes the ARN-Ig L chain, the VVHt, which produces the ARN-Ig H chain, and

the VVTG186, which does not produce any recombinant protein.

rVV expressing the murine Ig genes. Three rVV were used in this study and were generated by Transgene (Strasbourg, France) from the Copenhagen strain. The complete H and L chains of the monoclonal antibody (mAb) CO17-1A^{23,24} were introduced into the genome of the VV. To obtain the pTG1142 plasmid, the cDNA coding for the H and L chains were introduced into the plasmid pTG186poly²⁵ downstream of two copies of the 7.5 early/late vaccinia promoter. The two expression blocks were placed in opposite orientations in order to avoid homologous recombination between the two copies of the promoter. For the construction of the pTG2179, the cDNA coding for the H chain of the mAb CO17-1A was cloned in phase, upstream of the sequence coding for the transmembrane and intracytoplasmic domains of rabies glycoprotein.²⁶ As for the pTG1142, the cDNA coding for the L and modified H chains were introduced into plasmid pTG186 downstream of the 7.5 vaccinia promoter, in opposite orientations. *In vitro* homologous recombination in primary chicken fibroblasts²⁷ of VV DNA with the plasmids pTG186poly, pTG1142, and pTG2179 resulted in the generation of VVTG186, VVTG1142, which synthesizes a secreted version of mAb CO17-1A, and VVTG2179, which produces a membrane-anchored version of the same mAb. The foreign genes inserted into the VV thymidine kinase (TK) gene are controlled by the 7.5 vaccinia promoter, and generate TK⁻ progeny. The expression of the mAb CO17-1A at the membrane of BHK-21 cells infected by VVTG2179 was verified by FACSscan analysis. The production of the mAb CO17-1A by BHK-21 cells infected by VVTG1142 was measured in the culture supernatant by enzyme-linked immunosorbent assay (ELISA).

Transfectoma cells

The rearranged VHDJH gene complex was cloned in the expression vector pRTM1 as described,²⁸ giving the pRTM1-ARN vector. The V κ J κ C κ gene complex was cloned in the expression vector pZeoSV (Invitrogen, San Diego, CA), resulting in the pZeoSV-ARN vector. The pRTM1-ARN and pZeoSV-ARN vectors were linearized and purified before being suspended in sterile TE (10 mM Tris-Cl, pH 8, 1 mM EDTA). Two micrograms of linearized DNA was electroporated into 1.5×10^7 P3X63.Ag8.653 cells

Figure 1. rVV expression vectors. The cDNA coding for the IgM-ARN truncated H chain (A) and for the κ light chain (B) were introduced into the VV sequence (VV-hindJ), downstream of the P7.5 early/late vaccinia virus promoter, using BamHI and SacI restriction sites. Striped boxes represent human sequences; the solid box represents the rabies transmembrane sequence (TS); AmpR designates the ampicillin gene resistance; the arrows show the directions of gene transcription.

as described.²⁸ Two days after electroporation, IgM-ARN transfectoma cells were selected in complete medium supplemented with 1 mg/mL geneticin (G418) and 0.5 mg/mL zeocin (Invitrogen), at pH 7.6. After selection, IgM-SMI²⁸ and IgM-ARN transfectomas were cultured in the same complete medium as the P3X63.Ag8.653 cells: RPMI-1640 (Life Technologies, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (DAP, Neuf-Brisach, France), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), and 100 µg/mL streptomycin (Life Technologies).

Cell lines

The 1083-17-1A murine hybridoma and the baby hamster kidney cells, BHK-21, were supplied by Transgene. The mAb CO17-1A producing hybridoma 1083-17-1A was obtained through fusion of BALB/c myeloma P3X63.Ag8.653 cells with splenocytes from BALB/c mice immunized with SW1083, which is a human colorectal carcinoma cell line.^{29,30} All cell cultures were maintained in the medium described above, except BHK-21 baby hamster kidney cells that were cultured in Glasgow medium (Life Technologies) containing 10% fetal calf serum (FCS).

Flow cytometric analysis

H97 FITC-labeled mouse antimouse-H-2D^d and H-2K^d, 14.4.4 FITC-labeled mouse antimouse I-E^{k,d,b,s}, FITC-labeled goat antihuman κ light chain (Caltag, Burlingame, CA), FITC-labeled goat antihuman μ heavy chain (Jackson Immunoresearch, West Grove, PA), and biotin-conjugated hamster antimouse Fas (Pharmingen, San Diego, CA), followed by FITC-labeled streptavidin (Caltag), were used for 15 minutes at 4°C to stain transfectomas and BHK-21-infected cells before being analysed in a Becton Dickinson FACScan, Mountain View, CA. For the cytoplasmic staining of IgM-ARN transfectomas, we used the Fix and Perm kit (Caltag) following the manufacturer's instructions.

Animal immunization and challenge

Six- to 8-week-old female BALB/c mice were purchased from Janvier (Le Genest-Saint-Isle, France). Mice were immunized three times at 14-day intervals, with 10⁷ pfu of one of the purified rVV. This was done by tail scarification or intraperitoneal (i.p.) injections with 5 or 200 µL, respectively, of PBS containing the respective rVV. One week after the last boost, scarified mice were subcutaneously (s.c.) challenged with 40×10⁶ IgM-ARN transfectomas or IgM-SMI control transfectomas, whereas i.p. immunized mice were injected by i.p. route. In the syngeneic mouse model, mice were challenged by s.c. injection of 20×10⁶ 1083-17-1A hybridoma or control cells. The mice were then monitored for tumor growth by measuring perpendicular tumor dimensions with calipers.

Lymphoproliferation assay

Spleen and lymph node cells were collected 7 days after the third immunization or after the IgM-ARN transfectoma challenge, and CD4+ cells were purified with a MiniMACS

magnetic cell sorting kit (Miltenyi Biotec, Auburn, CA) using microbeads conjugated with anti-CD4 mAb (Miltenyi Biotec). Stimulating cells were resuspended at 5×10⁷ cells/mL and treated with mitomycin C (Sigma, St. Quentin Fallavier, France) at a final concentration of 50 µg/mL for 20 minutes at 37°C. Cells were then washed three times in RPMI-1640 before being suspended at a concentration of 10⁵ cells/mL in culture medium. Purified CD4+ or CD4− effector cells (E) were co-cultured for 5 days at 37°C with 100 µL of stimulating cells (S) treated with mitomycin C at various E/S ratios. Cultures were pulsed with ³H-labeled thymidine (1 µCi/well) (Du Pont/NEN, Les Ulis, France) for the final 18–24 hours of incubation. Tests were run in triplicates, in round-bottom 96-well polystyrene plates. Cells were collected with a cell harvester, and incorporated radioactivity was measured by liquid scintillation spectroscopy. The stimulation index was calculated for each triplicate by dividing the mean radioactivity of experimental-stimulated cells with that of negative control-stimulated cells.

Cytotoxicity assays

CD4+ or CD4− cells were cultured in the presence of mitomycin C-treated IgM-ARN or IgM-SMI transfectoma cells, at ratios of 10/1 or 100/1, with or without IL-2 (Boehringer Mannheim Biochemicals). After 5 days of incubation, cells were harvested and various numbers of effector cells were added to 10⁴ target cells that were labeled with Na⁵¹CrO₄ (CIS Biointernational, Gif-sur-Yvette, France) in round-bottom 96-well plates. Four-hour cytotoxicity assays were performed in triplicates and specific lysis was calculated as follows: % specific lysis = (experimental cpm − spontaneous cpm) / (maximal cpm − spontaneous cpm) × 100. Spontaneous lysis was less than 10% of specific lysis.

Evaluation of the anti-idiotypic humoral response by a cytofluorometric test

Blood samples were taken from VVHt- and VVTG186-immunized mice 7 days after the third immunization or 7 days after the inoculation of the IgM-ARN transfectoma cells. The presence of anti-idiotypic antibodies was tested by determining the capacity of the sera to bind to surface IgM expressed by the leukemic B cells of the patient ARN, after neutralization of the Ab directed against the Cμ human region. Leukemic cells expressing sIgM, from other CLL patients, were used as controls. Serum dilution of 1/50 was incubated for 20 minutes at room temperature with 10 µg/mL human IgM (Sigma). Fifty microliters of this mixture was added to 2×10⁵ CLL lymphocytes for 30 minutes at 4°C. After washing, cells were allowed to react with an FITC-labeled rabbit antimouse IgG (F(ab')₂) antibody (Jackson Immunoresearch) for 15 minutes at 4°C. Cells were washed again and analysed using a Becton Dickinson FACScan.

Evaluation of the anti-idiotypic humoral response using an ELISA assay

Plates were coated with 1 µg/mL goat antihuman (Fc5μ) (Jackson Immunoresearch) and incubated for 1 hour at 37°C.

Figure 2. VVHt immunization induces protection against transflectoma growth. BALB/c mice were immunized by tail scarification three times at 14-day intervals. Seven days after the last boost, the mice were challenged by s.c. inoculation of 40×10^6 IgM-ARN transflectoma cells (**A**, **B**, **C**, **D**) or IgM-SMI transflectoma cells (**E**). Tumor size was estimated as the product of perpendicular diameters. (**A**) Sixteen nonimmunized control mice. (**B**) Thirteen control VVTG186-immunized mice. (**C**) Six mice immunized with VVL expressing IgM-ARN κ light chain. (**D**) Thirteen mice immunized with VVHt expressing IgM-ARN H chain. (**E**) Ten mice immunized with VVHt before being challenged with IgM-SMI transflectoma cells. The number of mice bearing tumors (nt) and the number of mice without tumors (no), 10 days after challenge, are indicated.

After PBS-BSA 1% saturation, 50 ng/mL of human IgM (Sigma) or purified IgM-ARN was added and incubated overnight at 4°C. Suitable dilutions (1/50, 1/250, 1/1000) of the serum samples described above or 1 µg/mL of a control mouse antihuman IgM (Jackson ImmunoResearch) was added and incubated for 1 hour at 37°C. Bound antibodies were then detected with peroxidase-conjugated goat antimouse IgG (H+L) (Jackson ImmunoResearch) at 16 ng/mL for 1 hour at 37°C. In order to determine the presence of anti-idiotypic antibodies, IgM-ARN absorbance values were compared to total human IgM absorbance values.

In vivo blocking assays

Fifty microliters of rat antibodies in ascite form directed against mouse CD4 (YTS177.9.6.1)³¹ and mouse CD8 (YTS105.18.10)³¹ was administrated by i.p. route in VVHt-immunized mice following the schedules in Figure 6, A and B. For NK depletion, 30 µL of rabbit antiasialo GM1 (Wako BioProducts, Neuss, Germany) was injected i.p. in immunized mice (Fig 6C). Immunized mice were also treated with rat antimouse γ-interferon (IFN-γ) mAb (R46A2, 1 µg/mouse) (Pharmingen) (Fig 6D) or with hamster antimouse FasL mAb (MFL1, 2 µg/mouse)³² (Fig 6E). All mice were challenged with 40 × 10⁶ IgM-ARN transfectoma cells.

Statistical analysis

P values were calculated with Fisher's exact test.

RESULTS

Production of the rVV

In vitro homologous recombination of VV DNA (Copenhagen strain; see *Materials and Methods*) with pVVHt and pVVVL, in primary chicken fibroblasts, resulted in the

generation of the viruses VVHt and VVL, respectively. After *in vitro* co-infection of BHK-21 hamster cells with VVHt and VVL, the IgM-ARN was detected at the cell membrane by FACScan analysis (data not shown). When BHK-21 cells were infected *in vitro* with VVL alone, the human L chain was secreted in the supernatants (5 µg/mL), but when the cells were infected with VVHt, no H chain was detectable in the supernatants or at the cell membrane, leading to the conclusion that the protein was intracellularly processed. In order to design an efficient minimal immunization procedure, we tested VVL and VVHt VVs, separately.

Tumor challenges with IgM-ARN producing transfectomas

We produced transfectoma cells by electroporating the IgM-ARN expression vectors (pRTM1-ARN and pZeoSV-ARN) into nonsecreting murine myeloma P3X63Ag8.653 cells, as described.^{28,33} The transfectomas were shown to express low levels of class II MHC molecules (I-E^d) and high levels of class I MHC molecules (H-2D^d and H-2K^d) (data not shown). They produced cytoplasmic µ and κ chains, secreted intact IgM-ARN, but did not express it at the cell surface (data not shown). These transfectoma cells, when s.c. or i.p. injected into syngeneic BALB/c mice, produced measurable tumors within 7–10 days in at least 75% of cases (Fig 2A). When the cells were s.c. injected, the tumors expanded locally but did not metastasize. From day 15, approximately 20% of the tumors underwent necrosis associated with cutaneous ulceration. The i.p. cell injection resulted in the rapid appearance of solid tumors in the peritoneal cavity. The mice were immunized three times at 14-day intervals, with the control VVTG186 or with VVHt expressing the transmembrane H chain or with VVL expressing the L chain.

Figure 2 shows complete protection against tumor growth of the IgM-ARN transfectoma cells in mice immunized with VVHt expressing the IgM-ARN H chain (Fig 2D), but not with the control VVTG186 (Fig 2B) (*P*=.0005). Only

Figure 3. Lack of cellular responses detectable *in vitro* after VVHt immunization. Mice were immunized by tail scarification with VVHt or with the wild-type virus VVTG186. Seven days after the third immunization or after the IgM-ARN transfectoma inoculation, spleen and lymph node cells were harvested. **(A)** Lymphoproliferative response: Effector cells were co-cultured with mitomycin C-treated IgM-ARN transfectomas, or with IgM-SMI control transfectomas, or with allotypic control cells (C57BL/6 mice splenocytes). Proliferation was assessed by [³H]thymidine incorporation and shown as mean stimulation index

(SI) for each triplicate well (see *Materials and Methods*): Open histograms designate the SI when the experimental-stimulating cells are the IgM-ARN transfectomas and grey histograms designate the SI when the experimental-stimulating cells are the allotypic control cells. **(B)** Cytotoxic response: Lytic activity was assessed against IgM-ARN transfectomas (◆), IgM-SMI transfectomas (■), and C57BL/6 lymphoblasts (alloreactive control) (△). These experiments were carried out using the splenocytes of a VVHt-immunized mouse and were representative of the two groups of immunized mice after or before tumor challenge. The same results were obtained using the lymph node cells.

partial, nonsignificant protection was observed using the L chain construct ($P=.6$) (Fig 2C). No protection was observed in mice immunized with the ARN H chain construct and injected with transfectoma cells expressing a human IgM with different variable regions (IgM-SMI)²⁸ (Fig 2E). Mice injected i.p. with tumor cells were similarly protected by the VVHt virus (data not shown) ($P=.005$). These experiments show clearly that the tumor protection is complete and specific to the IgM-ARN H chain variable region.

In vivo tumor protection is not associated with in vitro detectable specific T-cell proliferation or cytotoxicity

Up until now, the mechanisms of tumor growth protection in different murine models of B-cell malignancies and in human lymphomas have only been partially investigated and have given contrasting results depending on the model and on the immunization protocol. However, both specific T-cell lymphoproliferation and humoral responses have been described. Despite the *in vivo* efficiency of our H chain VV construct, we were unable to detect a significant increase of T-cell proliferation when measuring [³H]thymidine incorporation into splenic or lymph node T cells in the presence of mytomicin C-treated IgM-ARN transfectoma cells (Fig 3A). We also failed to show any specific T-cell cytotoxicity (chromium release assay) when effector cells (CD4+ or CD4- cells) were cultured with or without IL-2 for 5 days in the presence of the target cells (Fig 3B). The harvesting of the effector cells after the last immunization or after the tumor challenge did not affect these results.

Antibodies against the tumor Ig are not detectable

Concerning the B-cell response in immunized mice, the antibodies that would be required for blocking experiments are not available, but cytofluorometric analysis of ARN leukemic cells (expressing the IgM-ARN at the membrane) or of control leukemic B cells incubated with the sera of the animals did not show a clear specific antibody response against IgM-ARN (data not shown). In the same way, the ELISA assay did not highlight significant quantities of antibodies directed against the IgM-ARN variable regions (data not shown).

CD4, but not CD8, T cells are critical for tumor protection

In order to determine which effector cells were engaged in the tumor protection, we performed *in vivo* mAb blocking experiments prior to the tumor challenge of immunized mice. When mice were injected with blocking amounts of anti-CD4 mAb (see the experiment schedule in Fig 4), the tumor protection was abolished (Fig 5A) ($P=.0005$). On the contrary, the anti-CD8 blocking assay failed to reverse significantly the protection against the tumor growth (Fig 5B) despite an efficient CD8 binding for 15 days (Ref.³⁴ and data not shown).

Considering the absence of CD8 T-cell requirement in the observed protection, we then tested the effect of NK depletion. It is known that treatment of mice with antiasialo GM1 antibodies³⁵ results in a total abolition of cytotoxic

A (anti CD4)

B (anti CD8)

C (anti asialo GM1)

D (anti IFN-γ)

E (anti FasL)

Figure 4. Blocking treatment schedules in VVHt-immunized mice. Mice were immunized as described by tail scarification and challenged (downward arrows) 7 days (C, D, E) or 10 days (A, B) after the last immunization with 40×10^6 IgM-ARN transfectoma cells. They were treated i.p. (upward arrows) with: (A) 50 μ L of rat ascite directed against mouse CD4 (YTS177.9.6.1) 7 and 3 days before the challenge and 4 days after; (B) 50 μ L of rat ascite directed against mouse CD8 (YTS105.18.10) 4 days before and 1 day after the challenge; (C) 30 μ L of rabbit antiasialo GM1, 1 day before and 5 days after the challenge; (D) 500 μ g/mouse of rat antimouse IFN- γ mAb (R46A2) on the day of the challenge and 250 μ g/mouse twice 3 and 6 days later; (E) 0.5 mg/mouse hamster antimouse FasL mAb (MFL1) on the day of the challenge and every following third day over a period of 9 days.

Figure 5. *In vivo* blocking experiments in VVHt-immunized mice. Tumor development was monitored by measuring perpendicular tumor dimensions in VVHt-immunized mice injected with IgM-ARN transfectomas and treated with: **(A)** antimouse CD4, **(B)** antimouse CD8, **(C)** antimouse asialo GM1 mAb, **(D)** antimouse IFN- γ mAb, and **(E)** antimouse FasL mAb. The number of mice bearing tumors (nt) and the number of mice without tumors (no), 10 days after challenge, are indicated.

activity against YAC-1 target cells, but it also has some effects on CD8 cytotoxic activity. In VVHt-immunized mice, antisialo GM1 treatment almost completely abolished the tumor protection (Fig 5C) ($P=.0005$), indicating that NK cells and/or macrophages could be involved in the effector mechanisms of protection.

Tumor protection is dependent on IFN- γ and partially on Fas-Fas ligand (Fas-FasL) interaction

Activated CD4 T cells secrete IFN- γ , which is capable of stimulating NK cells that can in turn produce IFN- γ

independently from cytotoxicity.³⁶ Sixteen mice were VVHt-immunized; three were treated with an anti-IFN- γ mAb. We observed the appearance of tumor growth in these three animals as was the case in nonimmunized mice (Fig 5D) ($P=.0018$).

An approach of the molecular mechanism leading to tumor protection in VVHt-treated mice was finally investigated. We decided to test the implication of Fas-FasL interaction because it has been shown to be involved in antitumor activity in different models³⁷⁻⁴⁰ and to enhance the sensitivity of tumor cell lines to Fas-mediated apoptosis.⁴¹ Because IgM-ARN transfectomas (tumor

Germline J558	Q	V	Q	L	Q	Q	S	G	A	E	L	V	R	P
CO17-1A	CAG	GTC	CAG	CTG	CAG	CAG	TCT	GGA	GCT	GAG	CTG	GTA	AGG	CCT
	---	---	---	t--	---	---	---	TT	---	---	---	---	---	---
								L						
Germline J558	G	T	S	V	K	V	S	C	K	A	S	G	Y	A
CO17-1A	GGG	ACT	TCA	GTG	AAG	GTG	TCC	TGC	AAG	GCT	TCT	GGA	TAC	GCC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---
CDR1														
Germline J558	F	T	N	Y	L	I	E	W	V	K	Q	R	L	G
CO17-1A	TTC	ACT	AAT	TAC	TTG	ATA	GAG	TGG	GTA	AAG	CAG	AGG	CTT	GGA
	---	---	---	---	---	---	---	---	---	---	---	---	-C-	---
													P	
CDR2														
Germline J558	Q	D	L	E	W	I	G	V	I	N	P	G	S	G
CO17-1A	CAG	GAC	CTT	GAG	TGG	ATT	GGA	GTG	ATT	AAT	CCT	GGA	AGT	GGT
	---	-G-	---	---	---	---	--g	---	---	---	---	---	---	---
		G												
Germline J558	G	T	N	Y	N	E	K	F	K	G	K	A	T	L
CO17-1A	GGT	ACT	AAC	TAC	AAT	GAG	AAG	TTC	AAG	GGC	AAG	GCA	ACA	CTG
	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Germline J558	T	A	D	K	S	S	S	T	A	Y	M	Q	L	S
CO17-1A	ACT	GCA	GAC	AAA	TCC	TCC	AGC	ACT	GCC	TAC	ATG	CAG	CTC	AGC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Germline J558	S	L	T	S	D	D	S	A	V	Y	F	C	A	R
CO17-1A	AGC	CTG	ACA	TCT	GAT	GAC	TCT	GCG	GTT	TAT	TTC	TGT	GCA	AGA
	---	---	---	---	---	---	---	---	--C	---	---	---	---	---
CDR3														
Germline DSP2.9	N	G												
CO17-1A	GAT	GGT												
	---	---												
Germline JH3		A	W	F	A	Y	W	G	Q	G	T	L	V	
CO17-1A		GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	
		C--	---	---	---	---	---	---	---	---	---	---	---	
		P												
Germline JH3	T	V	S	A										
CO17-1A	ACT	GTC	TCT	GCA										
	---	---	---	---										

Figure 6. Alignment of the CO17-1A heavy chain variable region nucleotide sequence with VHJ558, DSP2.9, and JH3 germline sequences. Nucleotide similarities are indicated by (—), silent mutations by small letters, and replacement mutations by capital letters. The location of the CDRs is indicated.

cells) express Fas on the cell surface (data not shown), we treated VVHt-immunized mice with an anti-FasL mAb able to block Fas–FasL interaction.³² As shown in Figure 5E, the treated mice developed a significantly higher number of tumors after the challenge, but the abolition of the tumor protection was not complete ($P=.00237$). These results could be related either to an incomplete blocking of Fas–FasL interaction or to the existence of another molecular mechanism of tumor protection.

rVV expressing minimally mutated mouse Ig is also able to protect against tumor growth

In order to test the vaccine approach in a syngeneic model, we deliberately decided to use rVV expressing an almost germline encoded Ig, CO17-1A. The Figure 6 shows the VH gene derived from the frequently expressed VHJ558 family, with only three replacement mutations. If the germline gene corresponding to the V κ

gene of CO17-1A is unknown, this V κ sequence is identical with the sequences reported for natural autoantibodies O1, O4, and E7,⁴² providing strong evidence that the CO17-1A V κ segment is directly encoded by a germline V κ gene. The 1083-17-1A hybridoma was found to elicit tumors in 100% of naive animals when s.c. injected (20×10^6 cells to three groups of 10 immunized mice and one group of 10 unimmunized mice). From days 0 to 6, the tumors appeared in equal number in all groups, but by day 10 after tumor challenge, seven of the VVTG2179 (membrane-anchored form)-immunized mice were tumor-free and three still presented small tumors (Fig 7D). Seven of 10 of the VVTG1142 (secreted form)-immunized and 9 of 10 of the control VVTG186-immunized mice presented tumors (Fig 7, C and B) of the same size as the control unimmunized group (Fig 7A). There is a significant statistical difference, 10 days after tumor challenge, between the number of tumors in VVTG2179-immunized mice and in VVTG186-immunized animals ($P=.0022$). Furthermore, comparison of the tumor size in these two

Figure 7. Antitumoral response after tumor challenge. Nonimmunized mice (**A**) and mice immunized with VVTG186 (wild type) (**B**), with VVTG1142 (secreted form) (**C**), and with VVTG2179 (transmembrane form) (**D**) were s.c. injected with 20×10^6 1083-17-1A hybridoma cells at day 0. Tumor growth was followed up by measuring perpendicular tumor dimensions with caliper. The number of mice bearing tumors (nt) and the number of mice without tumors (no), 10 days after challenge, are indicated.

groups of mice similarly revealed a significant difference (Mann-Whitney test, $P=.0011$). Interestingly, the VVTG1142 expressing the secreted form of the Ig was unable to significantly affect both the number and the size of the tumors in immunized animals.

DISCUSSION

These experiments and results are directly linked to two central immunotherapy issues: finding a feasible procedure that would assure efficacious vaccination and a means of determining the relative importance of the different possible effector cells.

In the first model, the vaccination with the H chain construct alone was able to induce specific and complete protection against tumor growth of transfectomas expressing the same VH region. Indeed, the observed protection was specific to ARN VH because the mice were not protected when challenged with an unrelated SMI VH producing transfectoma cells. Considering the absence of secretion and of membrane presentation of ARN μ chain in BHK-21-infected cells, our results suggest that the VH region was intracellularly processed during the immunization procedure in a way to promote T-cell priming. Our first step was to test the capacity of the vaccination procedure to induce specific T-cell proliferation and/or T-cell cytotoxicity because both of these tests have been shown to correlate with tumor protection in different experimental models, and even in clinical trials.⁴³ In both the xenogeneic (Fig 3) and the syngeneic models (data not shown), we were unable to demonstrate any specific *in vitro* proliferative and/or cytotoxic responses in immunized animals, even after tumor challenge. These results are reminiscent of recently published data showing that DNA vaccination against the idotype of a murine B-cell lymphoma does not lead to specific lymphoproliferative and cytotoxic responses.⁴⁴ However, the lack of detectable *in vitro* cellular immune response does not necessarily mean that it does not exist *in vivo*: one can question the sensitivity of bulk cellular assays as opposed to limiting dilutions assays; we can also question the relevance of these *in vitro* tests versus *in vivo* experiments.

Our xenogeneic model was designed to avoid an anti-idiotypic B-cell response, which is known to be able to confer tumor protection.¹² Indeed, we did not observe any anti-idiotypic antibodies in the sera of immunized animals.

Anti-CD4 and anti-CD8 blocking data clearly demonstrate that CD4 T cells are crucial to the protection of the animals. The time dosing of the anti CD4 mAb favors the hypothesis that the effector arm of the immune response was blocked, as has already been pointed out by Greenberg⁴⁵ and more recently by Hung et al.⁴⁶ However, in our model, it seems that CD4+ T cells were not mainly acting at enhancing *in vivo* the protective effect of CD8+ CTL because blocking experiments with anti-CD8 mAb did not significantly inhibit the tumor protection. Because we were unable to detect any specific CD4+ T-cell cytotoxicity *in vitro*, we can hypothesize that the CD4+ T cells recruited

other effector cells such as NK cells, macrophages, or eosinophils.⁴⁶ In our model, both NK cells and macrophages could be implicated because anti-sialo GM1 antibodies abolished the tumor protection. The precise mechanism of action of CD4+ T cells in tumor protection is presently unknown, but seems complex and not limited to providing signals required for the priming of MHC I restricted CD8+ T lymphocytes. In the case of vaccination of irradiated tumor cells transduced to secrete GM-CSF,⁴⁶ it was shown that both Th1 and Th2 cytokines were involved in tumor protection. We tested only the blockade of IFN- γ , which, effectively, showed that the Th1 pathway was involved in the tumor protection. Apparently, these CD4+ T cells were able to drive effector cells that do not, by themselves, have the capacity for antigen-specific recognition to a specific target. IFN- γ could be central to the protection mechanism because: (a) it could be produced by both CD4 T cells and NK cells; (b) it is implicated in the Th0→Th1 differentiation; and (c) it increases FasL expression in activated cells.

Keeping in mind the goal of immunotherapy during B-CLL, where V regions are frequently germline-encoded, it was important to test the capacity of the vaccination to induce an antitumor protection in a syngeneic model where the molecular target (variable region of the Ig) was deliberately chosen being almost unmutated. In this situation, autoreactive T and B cells would undergo clonal deletion or functional anergy as a result of central or peripheral tolerance mechanisms. However, low-avidity T cells could escape these mechanisms and could be activated through the vaccination procedure. Our data confirm the feasibility of the rVV approach in inducing tumor protection in a syngeneic model. The efficiency of our vaccination procedure could then rely on different peculiarities: (a) the capacity of the transmembrane form of rVV to induce the presentation of V region-derived peptides, particularly originating from the H chain third complementary determining region (mainly not germline encoded); (b) the ability to activate low-avidity T cells able to react with self-peptides; and (c) finally, the possibility to promote Th1 differentiation of effector cells and IFN- γ production.

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