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Antibodies Elicited by Naked DNA Vaccination against the Complementary-determining Region 3 Hypervariable Region of Immunoglobulin Heavy Chain Idiotypic Determinants of B-lymphoproliferative Disorders Specifically React with Patients' Tumor Cells[1]

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ABSTRACT

Several reports have suggested that the mechanism of protection induced by antiidiotypic vaccination against low-grade lymphoproliferative disorders is likely to be antibody mediated. Here we test the hypothesis that DNA vaccination with the short peptide encompassing the complementary-determining region 3 hypervariable region of immunoglobulin heavy chain (VH-CDR3) may elicit a specific antibody immune response able to recognize the native antigens in the form required for therapy. As a test system, we used the VH-CDR3 sequences derived from two patients with non-Hodgkin's B lymphomas (PA, AS) and one patient with hairy cell leukemia (BA) to immunize outbred Swiss mice. This experimental model could mimic a clinical setting in which different patients present distinct HLA haplotypes. Individual tumor-specific VH-CDR3 sequences were amplified by a two-step procedure and directly cloned into multigenic plasmid vectors (pRC100 and derived) with and without mouse interleukin 2 (mIL-2). Each tumor-specific sequence was characterized by sequencing. Female Swiss mice were vaccinated i.m. with plasmids expressing the tumor-specific VH-CDR3 sequence alone (pRC101-PA), mIL-2 plus the VH-CDR3 sequence (pRC111-PA), or a different unrelated antigen (NS3 of hepatitis C virus; pRC112), the sole mIL-2 (pRC110), and the empty plasmid (pRC100). Boost injections were performed at 3 and 16 weeks from the first vaccination, and sera were drawn before each vaccination and at 6, 9, and 19 weeks. Induction of anti-VH-CDR3s antibodies in the sera and their ability to recognize native antigens on patients' tumor cells were evaluated by FACS analysis. Up to 56% ($n = 25$) of mice vaccinated with pRC111-PA plasmid and 20% ($n = 15$) of mice vaccinated with pRC101-PA developed a specific immune response that was maintained throughout 19 weeks of observation in 40% of pRC111-PA-vaccinated mice. No response was detected in sera obtained from mice vaccinated with the other plasmids ($n = 45$). pRC111-PA injection s.c. was less effective (13%, $n = 15$) than i.m. injection (53%, $n = 15$).

Indeed, we demonstrated that antibodies elicited by naked DNA vaccination against three different patient-derived VH-CDR3 peptides (pRC111-PA or BA or AS) readily reacted with binding epitopes on the idiotypic proteins expressed on the surface of tumor cells derived from each patient; 60, 40, and 40% of, respectively, PA-, BA-, and AS-vaccinated mice developed specific antibodies. No cross-reactivity was detected among the three different CDR3s against tumor cells derived from the other two patients.

The outbred mouse strategy confirmed the significant matching potential of three different VH-CDR3 peptides to be efficaciously presented through different MHCs. We conclude that individual VH-CDR3 DNA vaccination can result in a potentially effective specific immune response against non-Hodgkin's B lymphoma cells by a rapid and low-cost therapeutic approach.

INTRODUCTION

B cell lymphomas may represent ideal models for experimental tumor immunotherapy. In fact the idiotypes of the Ig[3] (corresponding to the Ig hypervariable regions) displayed on their cell surface represent attractive tumoral antigens because they are tumor specific, belong to a well-known family of molecules, and it has been demonstrated that idiotype-specific immune response can be obtained also for self Igs. However, the efficacy of the antiidiotypic vaccination is likely to be more effective in some histiotypes than in others. Low-grade B cell lymphomas, such as those with a small lymphocytic or follicular histiotype, are expected to represent ideal targets because they (*a*) generally show an indolent behavior and are difficult to eradicate, (*b*) present a very low degree of clonal evolution, and (*c*) express surface Igs at high levels. Alternatively, antiidiotypic vaccination is expected to be less effective against lymphoblastoid and other high-grade lymphomas, because these types are very aggressive, may show a high degree of clonal evolution (30–40% in some cases), and in most instances do not express surface Igs. Although proved effective in experimental models (1) as well as in controlled clinical trials (2), the traditional approach to immunization (*i.e.,* s.c. immunization with the Ig borne by the B cell mixed with adjuvant) is hampered by the need of the high amounts of purified protein that must be prepared and certified for each case, within an appropriate time scale.

Several reports have indicated that the immunodominant epitopes of the clone-specific Ig lie within the hypervariable regions and mainly within the VH-CDR3 (3, 4). Furthermore, many observations suggest that immunization with whole protein may in some case produce an antibody response restricted to short linear epitopes, rather than recognizing the three-dimensional structure of the protein itself (5). This situation appears particularly frequent when antigens cross-reacting with self molecules are studied (6). Conversely, immunization with short peptides (8–20 aa residues long) can result in production of antibodies that recognize the corresponding linear epitope on a protein without need for conjugation to carriers, provided that the short peptide behaves like a complete antigen (*i.e.,* contains sequences able to bind Class II MHC molecules and to engage the T cell

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[3] The abbreviations used are: Ig, immunoglobulin; CDR3, complementary-determining region 3; VH, variable immunoglobulin heavy chain; IL-2, interleukin 2; mIL-2, mouse interleukin 2; FRW, framework; dNTP, deoxynucleotide triphosphate.

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receptor). Indeed, it was demonstrated that a short peptide encompassing the VH-CDR3 region of a human lymphoma-specific IgM was able to promote the *in vitro* proliferation of specific $CD4^+$ and $CD8^+$ cells capable of lysing the autologous lymphoma cells (7). As predicted by *in vitro* studies, the clinical relevance of these results was confirmed by the specific immune response obtained in a patient following vaccination with VH-CDR3 peptide and granulocyte-macrophage colony-stimulating factor (8).

The development of the method of vaccination by means of direct injection of naked DNA into the muscle or s.c., combined with the ability to easily identify and clone individual tumor-specific idiotypes, has improved the chances of exploiting these tumor antigens. This approach has already proved effective in inducing immune responses to several antigens mainly of viral origin (9), and it has been applied to therapy of experimental murine lymphomas where the Ig characterizing the lymphoma has been used for immunotherapy of the parental tumor via DNA-based vaccination (10–12). Such a method has proved effective in eliciting antiidiotype-specific immune responses when whole Ig, or its variable regions engineered to be expressed on nonself Ig (13), germline light chains (4), or toxin fusion protein (14), were used as encoded antigen. Nevertheless, if such a technology were to be transferred to clinical practice, a time-consuming work would still be needed for each patient. As outlined above, however, immunization with a peptide corresponding to the CDR3 region results in an immune response without need for further carrier allogeneic proteins (7). This approach avoids xenogenic or allogenic epitopes contained in the variable as well as in the constant region of the idiotypic immunoglobulin, enhancing the safety margin of this molecular approach. Finally, limited experimental data are available on the antiidiotype immunotherapy in outbred experimental models that can more closely reproduce the patient setting and immune response variability.

An important safety issue related to DNA vaccination is that purified double-stranded DNA itself is not immunogenic. Antinuclear or anti-DNA antibodies have never been detected, even after repetitive DNA injections, and no adverse, local, or systemic effects were identified (15). Several reports have demonstrated that naked DNA vaccination does not induce systemic lupus erythematosus in mice (although some anti-single-stranded DNA antibodies can be detected). Even more important, DNA vaccination did not alter the course of disease in NZB/NZW-*lpr* mice (16, 17).

We therefore tested the possibility of using the short peptide encompassing the VH-CDR3 as a target for eliciting a tumor-specific immune response via DNA-based vaccination. As a clear demonstration of the ability of anti-VH-CDR3s antibodies to recognize native antigens in the form required for therapy, FACS analysis was chosen to detect binding to patients' tumor cells bearing surface individual idiotypic Ig. Preimmune and immune sera obtained from DNA-vaccinated outbred Swiss mice were challenged with lymphoma cells derived from the corresponding patients.

In addition, to increase the immune response, we developed and used a multigene expression vector whose principal characteristic is the coexistence of two distinct, complete and differentially regulated transcription units, allowing the coexpression of mIL-2 and the VH-CDR3 Ig region (18).

MATERIALS AND METHODS

Human B-lymphoproliferative Cell Source. Two EBV-transformed cell lines (AS283A and PA682) derived from two non-Hodgkin's B cell lymphomas and leukemia cells (BA) from the peripheral blood of a variant-type hairy cell leukemia patient were used as sources of VH-CDR3 sequences. The WBC at diagnosis in the peripheral blood of the variant-type hairy cell leukemia patient was very high (130,000/mm^3), and the percentage of the leukemia cells was >95%. The cell lines used as a source material for the CDR3 sequences were clonal cells with origin from the primary lymphomas of the patients that was previously established (19). The identity of their CDR3 regions with the sequences used for DNA vaccination was established by comparing the sequence of the amplified fragments obtained from the tumor cells with the sequence of the plasmids used for vaccination (see below). The same cells were also used to determine the presence of antibodies reactive with the idiotypes in treated mice (see below).

VH-CDR3 Amplification. The amplification was performed in two steps, using the following family-specific PCR primers for human heavy chain CDR3 variable region: First step: upstream, $VH_{1\text{-}3\text{-}5\text{-}7}$ 5′-(C,G)AG GTG CAG CTG GTG (C,G)AG TCT-3′; VH_2 5′-CAG (G,A)TC ACC TTG AAG GAG TCT-3′; VH_4 5′-CAG GTG CAG CTG CAG GAG TCG-3′; VH_6 5′-CAG GTA CAG CTG CAG CAG TCA-3′; downstream, RH*mu* 5′-CAC GCT GCT CGT ATC CGA CGG-3′. Upstream primers were derived from the report of Deane and Norton (20). Downstream primer RH*mu* was designed to anneal with the 5′-terminus of the constant region *mu* of the human heavy chain. Second step: upstream (eFR_3 derived), eFW3 5′-*TTT* G/CTAGC ATG CAC ACG GC(C,T) (G,C)TG TAT TAC TGT-3′; downstream (eLJH derived), eJH 5′-*TAT* GC/GGCCGCTTA TTA TGA GGA GAC GGT GAC C-3′. Upstream and downstream primers for the second step PCR were derived from the work of Ramasamy *et al.* (21), with some modifications. As compared with published sequence, the eJH annealing sequence extends 2 nucleotides at the 3′-end of eLJH, and the eFW3 annealing sequence extends 1 nucleotide at the 5′-end of eFR_3, to improve specificity and PCR conditions. Moreover, both primers for the second step of PCR amplification included sequences for restriction sites (eFW3, *Nhe*I; eJH, *Not*I) (underlined), preceded by a short sequence (*italics*) for enhancing enzymatic cleavage efficiency (22).

RNA was first isolated from cell lines and frozen clinical samples, as described (23). cDNA was synthesized using a first-strand cDNA synthesis kit (Perkin-Elmer), murine leukemia virus reverse transcriptase (Perkin-Elmer) and random hexamers in a 20-μl final volume; reaction conditions were 23°C for 10 min, 42°C for 45 min, and 99°C for 5 min. Reverse transcriptase-PCR was performed in a final volume of 100 μl with 15 pmol of each primer, 50 μmol of dNTP, and 1 unit of AmpliTAQ (Perkin-Elmer); first step of amplification: 2 min at 96°C, followed by 5 cycles of 93°C for 1 min, 65°C for 30 s, 72°C for 30 s; and 30 cycles of 93°C for 30 s, 65°C for 30 s, 72°C for 30 s. The second step of all PCRs (nested PCR) was performed with the hot-start procedure; reaction conditions were 2 min at 96°C, followed by 10 cycles at 93°C for 30 s, 65°C for 30 s, 72°C for 15 s, and 20 cycles of 15 s for each step of denaturation, annealing, and polymerization.

The amplified fragments were sequenced by the dideoxy chain termination method using T7 DNA polymerase (Sequenase Version 2.0; USB). The CDR3 regions, the number of the somatic mutations present, as well as the germline sequences of the patients from which the leukemia/lymphoma cells were derived were established by comparing the sequence of the amplified fragments to the most homologous germline sequences of the Ig V genes present in the GenBank databases of the National Center for Biotechnology Information and in the V BASE sequence directory of the Medical Research Council Center for Protein Engineering (Cambridge, United Kingdom). The softwares used were, respectively, the BLAST program and the Mac Vector 6.0.1 software (Oxford Molecular Group PLC, Oxford, United Kingdom).

Plasmid Vectors, VH-CDR3 Cloning and Sequencing. Amplified variable regions and CDR3s were analyzed on a 2% SeaKem agarose gel (FMC, Rockland, ME). Bands were purified by QIAEX gel extraction kit (Qiagen, Inc., GmbH). The purified VH-CDR3 cDNAs were directly cloned into the *Nhe*I/*Not*I sites of one transcriptional cassette of pRC100-related plasmid vectors, with (pRC111) or without (pRC101) mIL-2 cDNA in the second transcriptional cassette (Table 1) (18). cDNA fragments were subsequently sequenced by dideoxy chain termination method using T7 DNA polymerase (Sequenase Version 2.0; USB).

Plasmid DNA Purification. Plasmid DNA was purified by Qiagen Plasmid Mega Kit (Catalogue No. 12181) and subjected to Triton X-114 extraction (24). Plasmid DNA preparations were evaluated following recommendations from FDA and other published works (25) and resuspended at 1.2 μg/μl in sterile 225 mM NaCl (1.5 × normal saline) in aliquots ready for use.

Animals and Immunization Protocols. Female Swiss mice about 8 weeks of age were used for immunization. Blood samples were obtained by tail

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Table 1 *DNA immunization constructs, encoded antigenic proteins, and immune modulator*

	Gene 1				Gene 2		
Plasmid	Promoter	Cloning sites	Gene	Source	Promoter	Cloning site	Cytokine
pRC100[a]	RSV[b]	*Nhe*L-*Not*L	N		CMV	*Xho*L	N
pRC101	RSV	*Nhe*L-*Not*L	VH-CDR3	BA HCL	CMV	*Xho*L	N
	RSV	*Nhe*L-*Not*L	VH-CDR3	PA B-NHL	CMV	*Xho*L	N
	RSV	*Nhe*L-*Not*L	VH-CDR3	AS B-NHL	CMV	*Xho*L	N
pRC110			N		CMV	*Xho*L	mIL-2
pRC111	RSV	*Nhe*L-*Not*L	VH-CDR3	BA HCL	CMV	*Xho*L	mIL-2
	RSV	*Nhe*L-*Not*L	VH-CDR3	PA B-NHL	CMV	*Xho*L	mIL-2
	RSV	*Nhe*L-*Not*L	VH-CDR3	AS B-NHL	CMV	*Xho*L	mIL-2
pRC112[c]	RSV	*Nhe*L-*Not*L	NS3	HCV strain H	CMV	*Xho*L	mIL-2

[a] Ref. 18.
[b] RSV, Rous sarcoma virus long terminal repeat promoter/enhancer; HCL, hairy cell leukemia; NHL, non-Hodgkin's B-cell lymphoma; BA/PA/AS, patients CDR3 sources; CMV, cytomegalovirus promoter/enhancer; N, none; HCV, hepatitis C virus; NS3, HCV nonstructural protein 3, 4403 to 4829-bp fragment.
[c] Ref. 27.

bleeds, and serum was stored at −80°C for subsequent assay. DNA injection was performed into the quadriceps muscle (i.m. injection) or as a s.c. bleb (s.c. injection), using an insulin syringe and 29-gauge × ½-inch needle (Becton Dickinson, Ref. No. 324804 microfine). Experimental groups received i.m. injections of 80 μg of pRC100-derived vectors encoding either only for CDR3 region (pRC101) or for mIL-2 only (pRC110) or for both (pRC111) (Fig. 1; Table 1). As controls, two other groups of mice were injected, under the same experimental procedure, with empty plasmid (pRC100) or with pRC100-derived vector encoding for mIL-2 and for a hepatitis C virus nonstructural antigen (NS-3; pRC112) (26). Two injections were performed respectively at the beginning of the experiment (T0) and three weeks after the first injection (T3). The third injection was performed 6 or 16 weeks after the beginning of experiments (T6 or T16). Mice were bled the day before each injection and at the following time points: T6, T9, and T19 (6, 9, and 19 weeks after the first injection, respectively).

IL-2 mRNA Detection. Total cellular RNA was extracted from excised muscles as described (23). 500, 50, and 5 ng of total RNA were reverse transcribed using 500 ng oligo(dT) primers (Life Technologies, Inc.), 1 mM dNTP (Promega), 10 U avian myeloblastosis virus reverse transcriptase (Promega), and 20 units of RNAsin (Promega), in 20 μl final volume. Incubation conditions were 42°C for 45 min and 99°C for 5 min. Mouse β-actin and mIL-2 sequences were amplified using 5 μl of each cDNA preparation, 1 unit of *Taq* polymerase (Promega), 2 mM $MgCl_2$ (Promega), 200 μM dNTPs, 20 pmol each primer, in 50 μl final volume. Mouse β-actin primers: forward, TgAgg CTCTT TTCCA gCCT; reverse, CTAgA AgCAC TTgCg gTgCA. mIL-2 primers: forward, CACTT CAAgC TCTAC AgCgg A; reverse, AAAAT TTgAA ggTgA gCATC C. Amplification was performed by 38 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min.

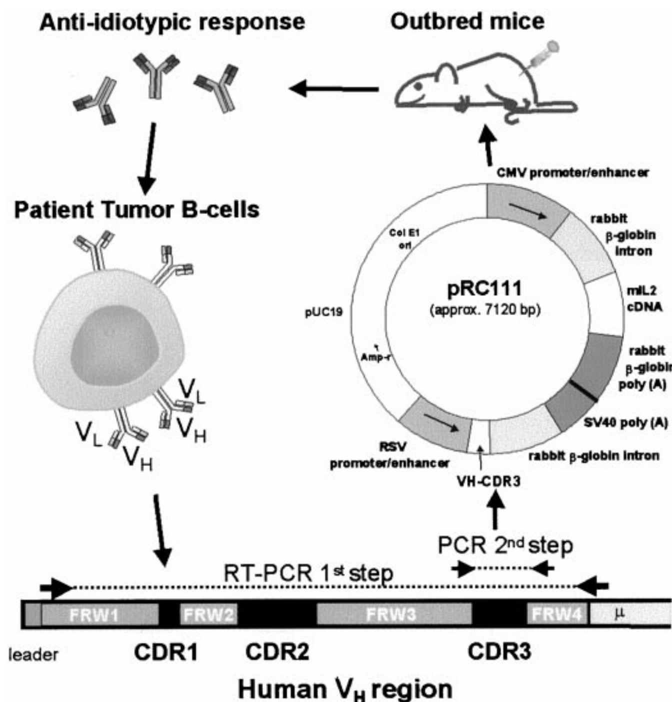

Fig. 1. Outline of the experimental strategy. Molecular rescue of VH-CDR3 sequences was accomplished from patient tumor B cells of different chronic lymphoproliferative disorders. Amplified sequences were cloned into a mammalian multigenic vector (pRC111) which independently coexpresses IL-2 and tumor-specific VH-CDR3 sequences. pRC111 vectors containing cloned VH-CDR3 sequences were injected in outbred mice. Sera obtained at different time points after DNA injection were challenged with the original patients' tumor B cells and tested by FACS analysis. *CMV,* cytomegalovirus; *RSV,* Rous sarcoma virus; *RT,* reverse transcriptase.

Anti-VH-CDR3 Antibody Detection. The production of antibodies by treated mice was assessed by FACS analysis, matching the sera with the EBV-transformed B cell lines (from patients PA and AS) or with thawed peripheral blood mononuclear cells (from patient BA), followed by incubation with FITC-conjugated goat antimouse antiserum (Coulter Clone), and detected by means of FACS (Profile II FACS; Coulter). All staining steps were performed in PBS supplemented with 5% FCS, 1% NHS, and 0.1% NaN_3 (staining medium). Cells (2×10^5) were incubated for 40 min in staining medium to reduce nonspecific binding, followed by a first step of staining with treated mouse serum diluted 1:50 or 1:20 in staining medium, in a final volume of 100 μl, for 30 min on ice. After three washes in staining medium, 10^5 cells were added as an internal control (only for PA and AS), and cells underwent a second step of staining with 1:500 FITC goat antimouse serum in a final volume of 100 μl. After further 30 min, cells were washed thoroughly and resuspended in staining medium, and their fluorescence was read using a Profile II FACS.

When performed using serum samples obtained before the first immunization (T0), FACS analysis usually gives a single peak, in which the internal control and the double-stained cells overlapped. Samples were selected as positive for anti-CDR3 antibody when two peaks were observed: the first one overlapped with that obtained with T0 sample and corresponded to the internal control (*i.e.,* the cells that were added after the staining with the immune serum, usually one-third of total cells); whereas the second, high fluorescence peak was due to those cells that had undergone the staining with "immune" serum (two-thirds of total cells). A 5-fold higher mean fluorescence value for the "high fluorescence peak" with respect to the internal control was chosen as threshold value of positivity for antiidiotypic antibody. For each individual mouse, all samples (T0–T19) were measured at the same time.

The specificity of the immune response developed against each individual VH-CDR3 sequence was evaluated matching sera from each group of animals with cells of the other patients and analyzed by FACS under the same technical protocol (p.e., sera from pRC111-PA-injected mice were matched with BA and AS cells).

RESULTS

Molecular Rescue of VH-CDR3 Sequences from Patient Tumor B Cells of Chronic Lymphoproliferative Disorders. The aim of this study was to demonstrate the effectiveness of the short hypervariable region (VH-CDR3) of the idiotypic Ig, expressed on B cells of chronic B-lymphoproliferative disorders, in allowing rapid cloning and spe-

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VH CDR3-BA sequence (Hairy Cell Leukemia)

1	2	3	4	5	6	7	8	9	10	11	12	13	14
M	H	T	A	V	Y	Y	C	A	R	V	L	Y	Y
ATG	CAC	ACG	GCT	GTG	TAT	TAC	TGT	GCG	AGA	GTT	TTG	TAT	TAC
15	16	17	18	19	20	21	22	23	24	25	26	27	28
D	F	W	S	G	Y	Y	I	S	N	Y	Y	Y	Y
GAT	TTT	TGG	AGT	GGT	TAT	TAT	ATT	TCT	AAT	TAC	TAC	TAC	TAC
29	30	31	32	33	34	35	36	37	38	39	40	41	42
Y	G	M	D	V	W	G	Q	G	T	T	V	T	V
TAC	GGT	ATG	GAC	GTC	TGG	GGC	CAA	GGG	ACC	ACG	GTC	ACC	GTC
43	44												
S	S												
TCC	TCA												

VH CDR3-AS sequence (chronic non-Hodgkin's B-lymphoma)

1	2	3	4	5	6	7	8	9	10	11	12	13	14
M	H	T	A	V	Y	Y	C	A	R	N	K	D	D
ATG	CAC	ACG	GCT	GTG	TAT	TAC	TGT	GCG	AGA	AAT	AAG	GAC	GAT
15	16	17	18	19	20	21	22	23	24	25	26	27	28
D	S	P	L	E	Y	W	G	R	G	T	L	V	T
GAC	TCC	CCT	CTT	GAG	TAC	TGG	GGC	CGG	GGA	ACC	CTG	GTC	ACC
29	30	31											
V	S	S											
GTC	TCC	TCA											

VH CDR3-PA sequence (chronic non-Hodgkin's B-lymphoma)

1	2	3	4	5	6	7	8	9	10	11	12	13	14
M	H	T	A	V	Y	Y	C	A	K	G	A	Q	G
ATG	CAC	ACG	GCT	GTG	TAT	TAC	TGT	GCG	AAG	GGT	GCG	CAG	GGC
15	16	17	18	19	20	21	22	23	24	25	26	27	28
A	S	L	G	K	A	Y	F	F	D	C	W	G	Q
GCA	TCA	CTT	GGT	AAG	GCC	TAC	TTC	TTT	GAC	TGC	TGG	GGC	CAG
29	30	31	32	33	34	35	36						
G	T	Q	V	T	V	S	S						
GGA	ACC	CAG	GTC	ACC	GTC	TCC	TCA						

Fig. 2. Nucleotide (*lower case*) and deduced amino acid (*upper case*) sequence of cDNA encoding human heavy chain CDR3 variable regions from three patients. *Bold*, part of the primer sequences. ▨, deduced CDR3 sequence (see "Materials and Methods").

cific immune response through naked DNA immunization. Two EBV-transformed cell lines (AS283A and PA682) derived from two non-Hodgkin's B lymphoma patients' cells, and one frozen peripheral blood sample from a hairy cell leukemia patient (BA), were used as sources of CDR3 sequences. Total RNA was purified from patients' cells and cDNA prepared using random hexamers. The amplification was performed in two steps. In the first step, the entire VH region was amplified (Fig. 1). 5′-Primers consisted of four degenerated primers identifying six family-specific sequences located in the 5′-end of the FRW1 of the human heavy chain V region (20). The 3′-primer annealed within the 5′-terminus of the constant region *mu* of the human heavy chain (see "Materials and Methods"). The amplified variable region was further amplified by nested primers annealing with the 3′-region of the FRW3 and with the 5′-region of the FRW4 (D–J) region. The use of RNA and of a two-step PCR procedure allowed high sensibility and specificity of yielded products. The individual VH-CDR3 fragments were directly cloned in pRC100-derived plasmid vectors (18) (Table 1) and sequenced (Fig. 2). These multigenic vectors incorporate two different transcription cassettes controlled by two independent promoters (cytomegalovirus and Rous sarcoma virus promoters/enhancers) and allows coexpression of a cytokine, mIL-2, together with a specific antigen, the individual VH-CDR3 peptide (pRC111) (Fig. 1).

IL-2 Is Produced at the Site of Naked DNA Injection. In a preliminary trial, eight 2-month-old Swiss mice were injected i.m. both with pRC111-PA vector coding for mouse IL-2 and VH-CDR3 from patient PA and with empty pRC100 plasmid in the contralateral muscles. Transcription was evaluated at the site of injection by semi-quantitative reverse transcriptase-PCR analysis, after 2 days and 1 week. Fig. 3 shows results obtained in four animals (named 1, 2, 3, 4) 1 week after injection of, respectively, pRC111-PA in the left quadriceps muscle and pRC100 in the contralateral muscle (named C2 and C4). Even if to different extents, IL-2 transcription was detected in all pRC111-PA-injected muscles, but no endogenous IL-2 expression was demonstrated in pRC100-injected contralateral muscles. The same results were obtained 2 days after injection (four animals; data not shown).

Naked DNA Injection of pRC111 Vectors Coding for the Individual VH-CDR3 Results in Immune Response against the Idiotypic Ig Expressed on Patient's Tumor Cells. Swiss mice 8 weeks old were injected i.m. with pRC101-PA (mIL-2$^-$) or pRC111-PA (mIL-2$^+$) vectors, in which VH-CDR3 from patient PA was cloned. As controls, three groups of mice were injected with either pRC100 (CDR3$^-$, mIL-2$^-$ vector), or pRC110 (CDR3$^-$, mIL-2$^+$ vector) (18) or pRC112 (encoding for hepatitis C virus nonstructural antigen NS-3, mIL-2$^+$) (26) under the same experimental schedule.

At various time points after DNA injection, mouse were bled, and the presence of antiidiotypic antibodies was tested by means of cytofluorimetric analysis. For each individual mouse, all samples were measured at the same time.

Fig. 4 shows the FACS analysis obtained with PA682 cells (patient PA) challenged by preimmune (Fig. 4*A*) and immune (Fig. 4*B*) serum samples of a mouse vaccinated with pRC111-PA vector. The cells stained with preimmune serum (T0) yield a single low fluorescence peak, in which the internal control (*i.e.,* baseline fluorescence obtained with the detecting antibody alone) and the double-stained cells overlapped (Fig. 4*A*). When the same cells were stained with the immune serum obtained 6 weeks after injection (T6), they distributed in two distinct peaks by FACS analysis (Fig. 4*B*): the first, low fluorescence peak overlapped with the preimmune, T0, peak and corresponded to the cells that were stained in the presence of detecting

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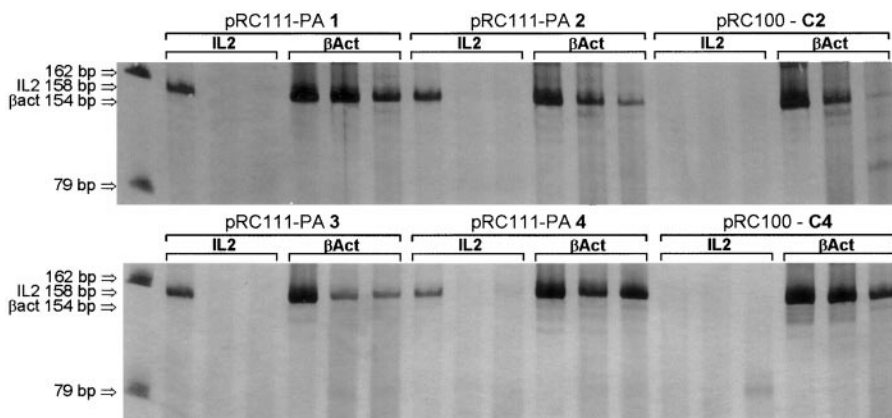

Fig. 3. Semi quantitative RT-PCR analysis of mIL-2 transcription in muscle of pRC111-PA-injected mice. Results are obtained using 500, 50, and 5 ng of total RNA prepared from four animals (named 1, 2, 3, 4) at 1 week postinjection. Mouse β-actin (βAct) and mouse IL-2 sequences were amplified as described in the text. Positive samples show a mIL-2-specific 158-bp amplified fragment. C2 and C4 (PA 2 and PA 4 contralateral muscles, respectively) were injected with empty pRC100 plasmid vector (negative control). First line: molecular weight marker (162 and 79 bp). βAct, mouse β-actin specific 154-bp amplified fragment.

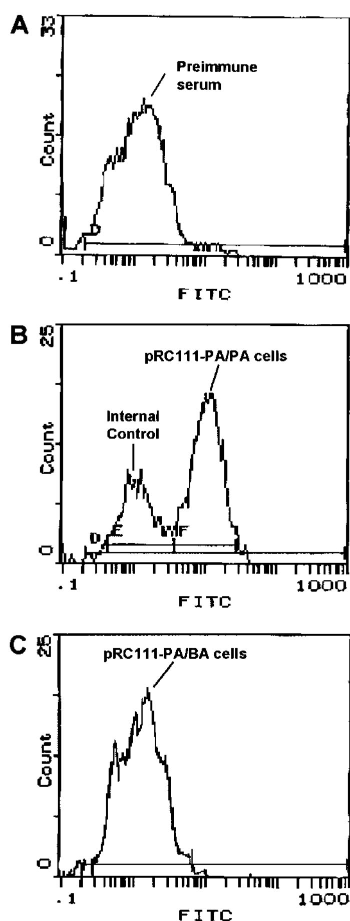

Fig. 4. Reactivity of Abs induced by DNA vaccine containing VH-CDR3 region from patient PA with the patient tumor cells, as assessed by immunofluorescence. Sera (diluted 1:50) from mice immunized with pRC111-PA were incubated with their target tumor cells (PA682) (*A* and *B*) or with tumor cells from patient BA (*C*). Control sera were from the same preinjected mice. After incubation, cells were washed, and bound antibodies detected by FITC-conjugated goat antimouse serum, using a FACScan. Results of a representative sample (PA) are displayed as peak presentations obtained with preimmune (*A*) and immune (*B*) serum (6 weeks after pRC111-PA injection). The internal control peak (*B*) shows the baseline reactivity of detecting goat fluoresceinated antimouse antibody alone. *C,* FACS analysis of pRC111-PA antiserum challenged with BA tumor cells.

antibody alone (internal control); the second, high fluorescence peak contained the double-stained cells, and therefore indicated that the T6 immune serum (pRC111-PA) was enriched in Igs recognizing (PA-specific) cell surface molecules. A dose response was found between the dilution of the sera and the intensity of fluorescence of reporter cells. As described in "Materials and Methods," samples that gave at least a 5-fold increase of mean fluorescence of stained cells with respect to the internal control cells were estimated as being positive for antiidiotypic antibody.

In a first set of experiments, we evaluated the ability of naked DNA encoding for the VH-CDR3 region to induce antiidiotypic antibodies, and we observed the effect of mIL-2 on this response. Only 20% of mice injected with pRC101-PA (IL-2^{-}) produced measurable amounts of antiidiotypic antibodies (Table 2). The injection of vector pRC111-PA (IL-2^{+}) resulted in production of Ig-specific antibodies in 56% of mice. Thus, IL-2 coexpressed with the antigen results in increase of efficiency of immunization. No specific antibodies were detected in mice injected with pRC100, pRC110, or pRC112 control vectors.

As shown in Table 2, the response was detected as early as 3 weeks after the first immunization, peaked soon after the second immunization, and persisted over a considerably long span of time, because it was still detected after approximately 5 months. Furthermore, no decrease of titer of specific Igs was observed (not shown). Interestingly, the coinjection of IL-2 increases the number of responding mice, but does not modify the kinetics of antibody production.

When we evaluated the overall efficiency of two different routes of DNA administration, we found that i.m. administration of pRC111 plasmid vector resulted in a stronger and more sustained immune response as compared with s.c. route (53% *versus* 13% responding animals, respectively; data not shown).

Anti CDR3 Responses Can Be Obtained with Different CDR3s. Two more points needed to be clarified to propose this approach for immunotherapy: (*a*) to demonstrate that such an approach can be successful for several different VH-CDR3; (*b*) to show that the antibodies elicited really recognize a "private" epitope of the Ig, to minimize the possibility to generate a systemic self-reactive disease. To address these points, in a new set of experiments, the VH-CDR3 from two other patients (AS and BA) were cloned. Swiss mice were immunized with the resulting vectors pRC111-AS or -BA, respectively, using the protocol described in Table 3. As shown in Table 3A, the rate of positive results obtained using three different CDR3s was similar. This observation confirmed that naked DNA immunization with vector pRC111 can result in anti-CDR3 response for several distinct CDR3s.

To address the second point, immune sera were also tested for their ability to recognize cells obtained from the other patients (*e.g.,* sera positive for anti-CDR3-PA response were also matched with cells from patients AS and BA, and *vice versa*). This experiment allowed us to check the fine specificity of the antibody response. No cross-reactivity toward the CDR3s different from the one used for immunization was found among sera that had tested positive (Fig. 4*C*; Table 3B). This result confirms that no response against frame regions of the CDR3s was elicited using this protocol and therefore that the risk of spreading of the immune response toward systemic self-reactivity is possibly low.

DISCUSSION

Our investigation addresses the question of whether different short peptides (30- to 40-mer) encompassing the VH-CDR3 hypervariable

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Table 2 *DNA injection schedule and immune response against non-Hodgkin's lymphoma cells from patient PA*

Three DNA injections were given at T0 (first); T3 (second), 3 weeks; and T16 (third), 16 weeks.

Plasmid	IL-2	Insert	Patients' cells	No. of animals	Immune animals (%)				
					3 wk	6 wk	9 wk	16 wk	19 wk
pRC 100	N	N	PA	15	0	0	0	0	0
pRC 110	Y	N	PA	15	0	0	0	0	0
pRC 112	Y	HCV NS3	PA	15	0	0	0	0	0
pRC 101-PA	N	PA VH-CDR3	PA	15	13	20	20	13	0
pRC 111-PA	Y	PA VH-CDR3	PA	25	45	56	56	40	40

Outbred Swiss mice were injected i.m. at T0 and then 3 (T3) and 16 (T16) weeks later with different plasmid constructs. Sera were collected at 3, 6, 9, 16, and 19 weeks after the first injection. Lymphoma cells from patient PA were challenged by mouse sera and analyzed by FACScan (see "Materials and Methods"). Results are expressed as percentage of positive animals. Y, N, yes or no; HCV, hepatitis C virus.

Table 3 *Specificity of anti-VH-CDR3 immune response versus lymphoproliferative cells from three patients (PA, AS, BA)*

Three DNA injections were given: T0 (first); T3 (second), 3 weeks; and T6 (third), 6 weeks.

	Plasmid[a]	IL2	CDR3	Patients' cells	No. of animals	Immune animals (%)		
						3 wk	6 wk	9 wk
A.	pRC 111-PA	Y	PA	PA	5	20	60	60
	pRC 111-AS	Y	AS	AS	5	0	40	40
	pRC 111-BA	Y	BA	BA	5	NT	40	40
B.	pRC 111-PA	Y	PA	AS-BA	5	0	0	0
	pRC 111-AS	Y	AS	PA-BA	5	0	0	0
	pRC 111-BA	Y	BA	PA-AS	5	0	0	0
C.	pRC 110	Y	N	PA BA AS	5	0	0	0

[a] A, percentage of sera from mice injected with pRC111-PA/AS/BA vectors reacting with patient tumor cells expressing the same VH CDR3 (PA/AS/BA); B, specificity of immune response against each VH-CDR3. Sera from animals injected with vector encoding each idiotype were challenged with patients' cells expressing the other two VH-CDR3; C, control mice: sera from 5 mice injected with CDR3-free vector (pRC110) were challenged with patients' cells expressing each of the three VH CDR3. Sera were collected at 3, 6, and 9 weeks after the first injection. Results are expressed as percentage of positive sera determined by FACS analysis. NT, not tested; Y, yes; N, no.

region of the lymphoma/leukemia surface Ig, expressed by direct i.m. injection of the corresponding minigenes, may be efficiently presented to the immune system of different outbred subjects to generate antibodies capable of reacting with patients' tumor cells exposing the specific idiotypic protein. In this context, CDR3 idiotypic vaccines may be applied in the immunotherapy of low-grade non-Hodgkin's B cell lymphoma/leukemia, with the aim to mobilize the patient immune system against residual tumor cells during disease remission (27). The xenogenic, outbred mouse model was decided to mimic the MHC-1 variability present in a clinical setting without immunizing human subjects.

Restriction to the individual CDR3 region excluded xenogenic or allogenic epitopes contained in the variable as well as in the constant region of the idiotypic Ig, greatly enhancing the safety margin when this approach is transferred in a syngenic context.

Two peculiar features of direct i.m. gene transfer, *i.e.,* the possibility of eliciting strong immune responses against coded antigens (11) and the chronic systemic delivery of therapeutic proteins (28, 29), can be combined for exploiting a more effective result (18). We assembled a double-gene plasmid vector for the coexpression of the specific individual CDR3 sequences and of an immunomodulating cytokine, mouse IL-2. Direct i.m. injection of cytokines encoding plasmids yields biologically active molecules that act locally and systemically at nontoxic doses (29, 30). This vector may further improve the safety by linking the expression of the cytokine to the expression of the foreign antigen in the same cells. This strategy ensures the production of the immunomodulating molecule only when and where needed, that is as long as the foreign antigen itself is produced.

In addition no anticytokine immune responses or consequent immune-suppression have been thus far detected. We believe that it is unlikely that T cells responsive to a protein (IL-2) that is involved in all immune responses and is present at high concentration any time T cells are activated could survive the induction of tolerance. To our knowledge, there is only one report showing the possibility to induce anti-IL-2 antibodies in mice, but the protocol required immunization with a form of the IL-2 that was truncated and contained amino acid substitution (31).

We found that up to 60% of outbred animals injected with each VH-CDR3/IL-2 coexpressing plasmid vectors mounted a significant, rapid immune response that lasted at least for 19 weeks after the first DNA injection. The number of responding animals declined up to 20% in the absence of IL-2 costimulation. More importantly, all VH-CDR3 immune animals developed antibody response able to recognize the entire idiotypic Ig exposed on the patient-derived lymphoma/leukemia cells, as demonstrated by FACS analysis. No cross-reactivity was found when immune sera of each VH-CDR3 were challenged with tumor cells of the other two patients. A possible interpretation of these results is that DNA vaccination by VH-CDR3 linear short peptides may induce production of antibodies which identify specific linear epitopes on the folded idiotypic Ig. The route of DNA immunization, too, may influence the type and significance of the immune response (32). Immunization with s.c. DNA resulted, in our experiments, less effective than i.m. injection.

The ability of DNA vaccination to stimulate specific antibody response in outbred mice by different nonsecreted tumor-derived CDR3 peptides, suggest the effective endogenous processing and presentation of the peptides in association with various MHC1s. It was demonstrated that peptides ranging between 17 and 44 amino acid length, either synthetic (33) or generated in cells from minigenes (34) may be processed for MHC1 presentation by cellular proteolytic pathways (35). Retargeting secreted proteins to the cytoplasmic localization may improve rapid proteolytic degradation (36). Short peptides are degraded to products of different average sizes, up to the length of class-1 presented peptides (8–9 residues) (37, 38). This assumption implicates the generation of different proteolytic end products which may differentially contribute to the immunogenic potential of the entire peptide (33).

These results apparently contrast with an earlier demonstration that antibodies induced by CDR3 peptide immunization fail to react with the native IgM present on the lymphoma cell surface (39). Presentation of endogenously synthesized proteins (or peptide) results in the production of an epitopic repertoire slightly different from the one generated by processing of exogenous protein (40). This effect may therefore favor the selection of the appropriate peptide/MHC class II complex to induce optimal activation of $CD4^+$ T cells. Furthermore, peptides synthesized in the cell do not display the set of chemical modification of active residues often needed for their chemical synthesis. Both these effect together may favor the transfer to B cells of more "naturally conformed" peptides (either by DC or by muscle cells) in tandem with optimal CD4 dependent help. Furthermore, antigen synthesized within the same antigen-presenting cells by DNA vaccines are processed and presented by MHC class I complex as linear peptides of 8–12 aa, to stimulate naive $CD8^+$ cells (41). As outlined above, muscle cells may cross-prime $CD4^+$ T cells and B cells by secreted antigen or by liberation of intracellular antigen due to $CD8^+$ cell-induced killing of antigen-expressing myocytes (42). These considerations support two different pathways of immune stimulation for peptide and DNA immunization. Due to these differences,

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DNA immunization may be more efficient in presenting short peptides by a conformation-independent mechanism.

Priming by i.m. DNA vaccination is performed by bone marrow-derived antigen-presenting cells, which are efficient at providing all of the necessary signals for T cells (43, 44). The muscle cells participate in the immunization mechanism as a reservoir for the antigen (45) and a persisting immune stimulus. The method of DNA vaccination and the form of DNA-expressed antigen may bias T cell help to primarily type 1 or type 2 (32). The proposed experimental model includes both i.m. injection and nonsecreted peptides which may direct the response to the T helper 1 pathway. Moreover, IL-2 expression may function by enhancing T cell-mediated immune response (46) and by improving antigen-specific T cell proliferation (47) as well as differentiation and Ig secretion of antigen-activated B cells (48, 49). In this context, antibody response may be generated by B cell stimulation obtained by peptides released from muscle cells during immune-mediated destruction of transfected muscle fibers (42).

Several reports have suggested that the mechanism of protection against low-grade lymphoma is likely to be antibody mediated (14, 50), possibly due to direct induction of apoptosis (51, 52). Very recently, experimental data confirmed that tumor-protective effects of DNA vaccination can be mainly ascribed to idiotype-specific humoral immunity (53), and still more convincingly prolonged survival in an ongoing clinical trial has been correlated with the induction of anti-idiotypic antibody responses (54). However, the nature of a protective immune response in already established low-grade B cell lymphomas is still debated (8, 55). In our experimental model, the idiotypic peptides are neither secreted nor injected i.v. but are directly produced in *in vivo* transfected cells, in combination with recombinant IL-2 expression. This mechanism may warrant for antibody production and cytotoxic response, without the impairment associated with the binding of antiidiotypic VH-CDR3 antibodies to the secreted peptide or to the injected peptidic vaccine (12, 56). These potential advantages may be now further investigated in a syngenic environment.

Even if the VH-CDR3 region of the idiotypic Ig is most variable and therefore most likely to be unique to this protein, most of the somatic mutations that may develop under the immune pressure are clustered in the CDR1, CDR2, and FRW3 regions of the idiotypic protein (57, 58). Due to the ease in identifying new VH-CDR3 idiotypic variants as well as in rapidly cloning and manufacturing clinical grade DNA vaccine preparations, the proposed protocol may anyway be reapplied in the follow-up of the treated patient.

In conclusion, the immune responses generated by naked DNA immunization in an outbred animal model prove the potential immunogenicity and safety of the VH-CDR3 minigene-encoded peptides and provide the basis for further studies and optimization of this therapeutic strategy on selected patients with B-lymphoproliferative disease, to ascertain the therapeutic beneficial effects in a clinical setting.

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