Autologous stem cell transplantation in juvenile idiopathic arthritis : regaining immunological tolerance and arresting disease progression

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Chapter 6

Vaccination with rabies to study the humoral and cellular immune response to a T-cell dependent neoantigen in man

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Abstract

We investigated the humoral (antigen-specific immunoglobulin isotypes, IgG subclasses, and avidity maturation) and cellular (antigen-specific *in vitro* proliferation) immune response in 18 healthy adult volunteers, following a primary and a single booster vaccination with the T-cell dependent neoantigen rabies administered at a 3-months interval. The IgG antibody titer showed a mean 31-fold increase (range 3-154) 4 weeks after the first vaccination and a memory response was observed after booster vaccination, *i.e.* high IgG titers, switch from IgM to IgG and IgA and increased antibody avidity. All healthy adults showed a rabies-induced proliferative response with a mean stimulation index of 45 (range 3.5- 200) after *in vitro* stimulation of PBMC obtained at 4 weeks after booster vaccination. The results obtained in this study provide a frame of reference for the interpretation of specific immune responses to the T-cell dependent neoantigen rabies in patients suspected of a primary or secondary immunodeficiency. Humoral and cellular immune responses to the rabies neoantigen provide complementary information on the condition of the immune system of an individual. Five patients diagnosed with a combined immunodeficiency were vaccinated using the same protocol and showed a number of abnormalities, either in the humoral or the cellular immune response to the rabies neoantigen.

Introduction

A sensitive parameter for testing the function of the adaptive immune system is to investigate the appearance of an antigen-specific humoral and cellular immune response after *in vivo* exposure to a T-cell dependent neoantigen (1). Inactivated rabies virus might be a useful neoantigen, because rabies virus is a neoantigen for most individuals and there is evidence that the immune response towards rabies virus is T-cell dependent (2,3). In contrast to other non-licensed neoantigens, *i.e.* α-helix pomatia haemocyanin (HPH), keyhole limpet haemocyanin (KLH) and bacteriophage ΦX174 (4-6), rabies vaccine is registered for application in humans by the Food and Drug Administration (USA). Hepatitis B virus is not considered to be a reliable antigen for testing immune competence because of the high frequency of nonresponders in healthy individuals (1). There are no contraindications for the administration of human diploid cell vaccine (HDCV) rabies vaccine and since the vaccine contains no live virus, it is considered to be safe in patients with humoral or cellular immunodeficiency (7-9) and in pregnancy (10). Many studies have been published concerning the immunogenicity and safety of prophylactic vaccination regimens (in individuals at high risk of infection) and therapeutic vaccination regimens (after exposure to a bite of a rabid animal) (11, 12). These so-called pre-exposure and post-exposure vaccination schemes consist of a series of subsequent rabies vaccinations, within a period of a few weeks. For prophylactic vaccination the World Health Organization (WHO) recommends a primary series of immunizations at day 0, 7, 28, and a booster at day 90, to achieve the WHO recommended protective antibody titer of ≥ 0.5 IU/mL (13). In most studies, levels of IgG anti-rabies antibodies have been determined following a minimum number of three vaccinations (11, 12). As a consequence of these practices, no information is available on the characteristics of the humoral and cellular immune response after a primary rabies vaccination followed by a single booster after several months. Furthermore, no data are available with respect to the IgG subclass distribution and the avidity of anti-rabies antibodies after a primary and a single booster vaccination. The aim of this study was to investigate the characteristics of the humoral (quantity and avidity of antibodies) and cellular (*in vitro* proliferation) immune response in healthy adult volunteers following a primary and booster rabies vaccination given at a 3-months interval. These data provide a frame of reference for the interpretation of specific immune responses to the T-cell dependent neoantigen rabies in patients suspected of a primary or secondary immunodeficiency. To relate our findings to the healthy group with such a patient group, the humoral and cellular responses of five patients suffering from a combined B- and T-cellular immunodeficiency, were studied using the same vaccination protocol.

Patients and methods

Study population

Healthy volunteers (n=18), aged 19 to 49 years, were studied, following written informed consent. The study was approved by the Committee on Medical Ethics of the Leiden University Medical

Center. Exclusion criteria were previous rabies vaccination, the presence of a disease affecting the immune system, and treatment with immunosuppressive drugs. Furthermore, five patients, of whom three siblings, diagnosed with a combined immunodeficiency, aged 4 to 13 years, were included in the study. All five had complains and clinical symptoms of recurrent bacterial ear-, nose-, and throat (ENT) infections. Their serum levels of IgG, IgA and IgM were below normal ranges for age (14). Antibody titers to the recall antigens tetanus toxoid and conjugated *Haemophilus influenzae* type b polysaccharides after booster vaccination were absent or severely decreased. In three of five patients a decreased *in vitro* proliferative response of peripheral blood mononuclear cells (PBMC) to αCD3 and tetanus toxoid was observed, and in three of five patients numbers of circulating B- and T-cells were decreased. All patients were under immunoglobulin replacement therapy and prophylactic antibiotics.

Vaccine

The rabies vaccine used was the human diploid cell vaccine (HDCV), manufactured by the Institute Pasteur Merieux MSD, Lyon, France (commercial lot number NO 976-8). The vaccine is prepared using the Wistar PM/WI38-1503-3M strain of rabies propagated on human diploid cells and inactivated by β-propiolactone. HDCV is a whole virus vaccine consisting of a nucleocapsid complex surrounded by a lipoprotein bilayer membrane with glycoprotein projections at the outer surface. The lyophilized vaccine, containing at least 2.5 IU inactivated virus, was stored at 4 °C and reconstituted with 1 mL diluent just before use.

Immunization and sampling protocol

One milliliter of HDCV was given intramuscularly into the deltoid region at day 0 and a booster vaccination was given with the same dosage 3 months later. Three of 18 volunteers received a third vaccination 1 year after the first vaccination. Blood samples were drawn at day 0, 7, 14, 28, 365 after the first, and at day 0, 7, 14, 28 after the second and third vaccination. At the time of blood sampling after rabies vaccination the subjects were interviewed to evaluate whether they had experienced any adverse effects related to the vaccination. Serum was stored at –20 °C until investigation. PBMC were obtained by Ficoll-Isopaque density gradient centrifugation and stored in liquid nitrogen until further analysis.

Antigen for in vitro assays

Rabies virus Pitman Moore (PM) strain (Wistar PM/WI-38-1503-3M) was propagated in primary dog kidney cells (DKCV), concentrated and purified by ultrafiltration and inactivated with β-propiolactone, as decribed by van Wezel *et al*. (15) and manufactured at the National Institute of Public Health and Environmental Protection, RIVM, Bilthoven, The Netherlands. One dose of lyophilized vaccine contains 350 µg protein and was dissolved in 1 mL distilled water. DKCV, used as coating antigen in ELISA, was compared with the fifth International Standard for Rabies Vaccine (Statens Serum Institut, Copenhagen, Denmark, supplied by NIBSC, Potters Bar, United Kingdom), which is commercially

available (16). The results obtained from ELISA assays using either the fifth International Standard for Rabies Vaccine or DKCV as coating material were comparable (data not shown).

Antibody quantification

The concentrations of IgG, IgG subclasses, IgA and IgM anti-rabies antibodies were measured by sandwich ELISA technique. For measurement of IgG, IgG subclasses and IgA, flat bottom microtiter plates (Costar, Cambridge, MA, USA) with high binding capacity were coated with 100 µL/well DKCV in PBS pH 7.5 (10 µg/mL), incubated for 3 hours at 37 °C followed by overnight incubation at room temperature. Subsequently, the plates were incubated with 150 µl/well PBS/0.05% Tween 20/2% caseine (PBS/T/C, Sigma, St. Louis, MO, USA) for 1 hour at 37 °C to block remaining binding sites and thoroughly washed three times with PBS/0.05% Tween (PBS/T). Each subsequent incubation step was performed at 37 °C in a humidified atmosphere and followed by washing steps with PBS/T. All dilutions were made in PBS/T/C. The plates were incubated with 100 µL/well serum dilutions for 2 hours. For detection of total IgG and IgA, the plates were incubated for 2 hours with either 100 µL/well goat-anti-human IgG (1:1500) or goat-anti-human IgA (1:1000), conjugated with alkaline phosphatase (Biosource, Camarillo, CA, USA). For detection of IgG subclasses, the plates were incubated for 2 hours with monoclonal mouse-anti-human IgG subclass specific antibodies (in a 1:10.000 dilution for IgG1 (WHO/IUIS HP 6188 (17), CLB, Amsterdam, The Netherlands), IgG2 (clone no. 36-127-2, TNO, Leiden, The Netherlands) and IgG3 (WHO/IUIS HP 6080, Nordic, Tilburg, The Netherlands), and in a 1:25.000 dilution for IgG4 (WHO/IUIS HP 6206, Nordic). After incubation with the monoclonal antibodies, the plates were incubated at room temperature overnight with 100 µL/well rabbit-anti-mouse Ig conjugated with alkaline phosphatase (RAMAP 1:750; DAKO, Glostrup, Denmark). After incubation with conjugated antisera the substrate PNP (4-nitrophenyl-phosphate disodium salt, Merck, Darmstadt, Germany) in 0.01 M DEA buffer, pH 9.8 (diethanolamine buffer, BDH, Poole, England) containing 150 mg/L $MgCl_2$.6H2O (Merck) was added (100 µL/well). The reaction was stopped by addition of 100 µL/well 3M NaOH (Merck). The absorbance was measured at 405 nm using an automatic spectrophotometer (Titertek Multiscan, Labsystems, Helsinki, Finland).

Using DKCV as coating antigen, quantification of rabies-specific IgM was hampered because of nonspecific binding resulting in high pre-immune values. For that reason, IgM anti-rabies was determined using a commercial ELISA kit, *i.e.*, PLATELIA RAGE that contained purified glycoprotein as antigen (BioRad, Hercules, CA, USA), according to the manufacturer's instructions. The kit was designed for measurement of IgG anti-rabies and was modified for the detection of IgM anti-rabies. Therefore, the plates were finally incubated with 100 µL goat-anti-human IgM conjugated with alkaline phosphatase (1:1000; Biosource, Camarillo, CA, USA), followed by substrate and stopping agent as described above. The second International Standard reference serum of the WHO containing 30 IU/mL of total IgG anti-rabies (18) (Statens Serum Institut, Copenhagen, Denmark, supplied by NIBSC, Potters Bar, United Kingdom) and an in house prepared secondary reference serum (total IgG anti-rabies 3.3 IU/mL) were used to express the IgG data in IU/mL. For IgG-subclasses anti-rabies and IgM and IgA anti-rabies no reference sera are available; therefore, our secondary reference serum was used. The secondary reference serum consisted of pooled sera of the 18 individuals obtained

at 2 and 4 weeks following the first rabies immunization, and values for IgM and IgA anti-rabies antibodies were arbitrarily set at 1 U/mL. Indirectly, IgG-subclass anti-rabies could be expressed in IU/mL by determination of the relative contribution of the IgG subclasses to the total IgG anti-rabies response. To establish the proportion of IgG subclasses, sera were absorbed with Protein A Sepharose® CL-4B (Pharmacia, Uppsala, Sweden) which specifically binds IgG1, IgG2 and IgG4. A column of 7.0 mL Protein A Sepharose CL-4B was used for processing, and IgG, IgG1 and IgG3 were determined in fractions of bound and unbound proteins by nephelometry (BN 100 Analyzer; Dade Behring, Marburg, Germany).

Antibody avidity

The avidity of IgG-subclass anti-rabies was measured by a modified elution ELISA (19), in which well-chosen dilutions of serum samples were allowed to interact with rabies DKCV coated on the wells. Subsequently, wells exposed to a distinct sample dilution, were incubated for 15 minutes at 37 °C with a variable molarity (range 0.5-4.5 M) of the chaotropic agent sodium thiocyanate (NaSCN) in PBS. IgG-subclass anti-rabies antibody levels were measured as described above. The relative avidity index (AI) is defined as the molarity of NaSCN at which 50% of the amount of IgG-subclass antibodies remained bound to the coated rabies antigen. Heterogeneity of avidity maturation was calculated from Shannon's coefficient (20) and the relative high affinity value (RHAV) was determined as described by Luxton (21).

Lymphocyte proliferation

Triplicate cultures of $1x10^5$ PBMC/well were performed in 96 well round-bottom microtiter plates (Costar, Cambridge, MA, USA) in a final volume of 200 µL RPMI 1640 glutamax 1 (Gibco, Paisly, Scotland) supplemented with 10% heat-inactivated pooled human AB serum, penicillin (100 U/mL; Gibco) and streptomycin (100 µg/mL; Gibco). Cells were cultured with variable concentrations of DKCV, range 0.005-50 µg/mL, at 37 °C and 5% CO2 for 4-7 days. Subsequently, 1 µCi/well ^{3}H-thymidine (Amersham, San Francisco, CA, USA) was added 18 hours before harvesting. ^{3}H-thymidine uptake of cultured PBMC was measured as counts/minute (cpm) of triplicate cultures by liquid scintillation counter (Wallac, Turku, Finland) and expressed as a stimulation index (SI) (ratio mean cpm DKCV/ mean cpm medium). Stimulation index ≥ 3.0 was considered as evidence of antigen-induced proliferation. In dose response curves, the highest median SI was achieved using a concentration of 0.5 µg/mL rabies antigen (data not shown). In time-course studies an optimal lymphocyte proliferation to rabies was measured after 5 days of culture (data not shown). As positive control, the proliferative capacity of the PBMC was tested by mitogenic and polyclonal stimulation, using phytohemagglutinin (PHA, 5 µg/mL Murex, Dartford, England) and anti-CD3 monoclonal antibody (αCD3). Therefore, microtiter plates (96 well flat-bottom microtiter plates; Costar) were coated with 100 µL αCD3 (0.1 µg/mL OKT3 in PBS, Ortho Clinical Diagnostics, Rochester, NY, USA) for 4 hours at room temperature. Triplicate cultures of 0.4 $x10^5$ PBMC/well were performed for αCD3 and PHA, followed by ^{3}H-thymidine incorporation at day 4. Medium was used as a negative control.

Table I. Concentration of Anti-Rabies Antibodies

	Total IgG			IgG1			IgG3			IgA			IgM		
	GMT (IU/mL)	95% CI	Significance	GMT (IU/mL)	95% CI	Significance	GMT (IU/mL)	95%CI	Significance	GMT (U/ml)	95% CI	Significance	GMT (U/ml)	95% CI	Significance
0	0.1	0.0, 0.2		0.0	0.0, 0.1		0.0			0.1	0.0, 0.5		0.1	0.0, 0.8	
1	0.1	0.0, 0.3	NS	0.0	0.0, 0.2	NS	0.0	0.0, 0.1	NS	0.1	0.0, 0.5	NS	0.2	0.1, 0.9	**
2	1.6	0.3, 7.7	***	1.2	0.2, 8.2	***	0.3	0.0, 2.5	***	0.9	0.2, 4.7	**	1.3	0.3, 5.2	***
4	1.9	0.4, 9.4	***	1.5	0.2, 10.1	***	0.4	0.1, 2.7	***	0.4	0.1, 1.4	**	0.7	0.2, 2.8	***
13	0.8	0.2, 3.1	***	0.7	0.1, 3.1	***	0.1	0.0, 0.4	***	0.2	0.1, 0.7	***	0.2	0.1, 0.8	*
14	15.7	3.2, 76.1	###	15.1	2.8, 82.0	###	3.5	0.4, 29.0	###	1.7	0.4, 6.5	###	0.6	0.2, 1.7	###
15	23.3	6.5, 83.2	###	23.6	5.7, 97.2	###	4.5	0.6, 34.0	###	2.5	0.5, 11.0	###	0.6	0.2, 2.4	###
17	18.1	4.9, 66.9	###	18.3	3.9, 85.3	###	2.5	0.3, 19.3	###	1.2	0.3, 5.8	##	0.4	0.1, 1.8	##
52	1.6	0.4, 5.5	^^^	1.4	0.4, 4.8	^^^	0.1	0.0, 0.5	^^^	0.4	0.1, 1.4	^^^	0.2	0.0, 0.6	NS

Note. Geometric mean titre (GMT) and 95% confidence interval (CI) of total IgG, IgG1, IgG3, and IgA, IgM anti-rabies at the indicated time-points after primary (week 0) and secondary (week 13) immunization with rabies vaccine.

Statistics: Paired *t* test (* compared to week 0/ # compared to week 13/^ compared to week 17): NS, not significant; */#/^ $p < 0.05$, **/##/^^ $p < 0.01$, ***/###/^^^ $p < 0.001$.

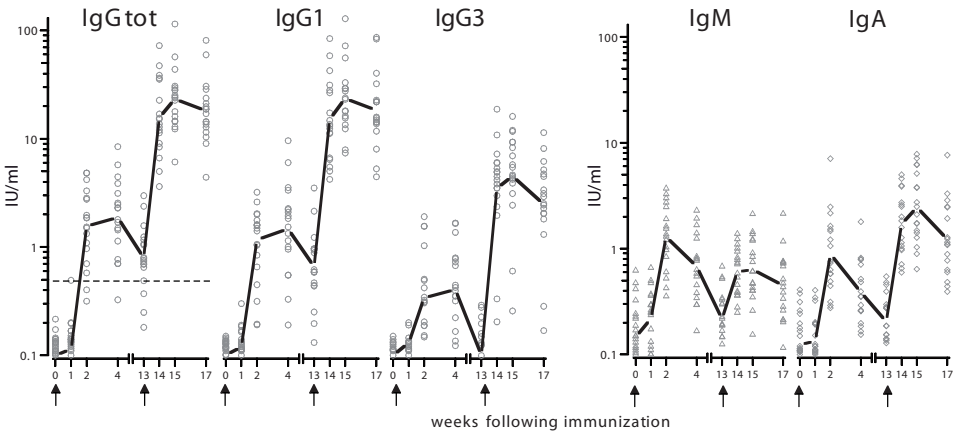

Figure 1. Total IgG, IgG1, IgG3 anti-rabies (in IU/mL) and IgM, IgA anti-rabies (in U/mL) following primary and secondary rabies immunization in healthy adults. Individual data and geometric mean titer (GMT) are depicted. *Arrows* indicate week of immunization. *Dashed line* represents WHO seroconversion level (IgG anti-rabies 0.5 IU/mL) (13).

Statistical analysis

Statistical comparisons were carried out using a computer software program (SPSS). The geometrical mean titer (GMT), expressed in IU/mL for IgG and IgG subclasses and in U/mL for IgA and IgM anti-rabies, with its 95% confidence interval (CI), were calculated for each time-point of sampling. Differences between time-points with respect to GMT values for all parameters were assessed by the paired Student's *t* test. The differences between a primary and secondary immune response, *i.e.,* concentration and kinetics of immunoglobulin levels, isotype switch and avidity maturation, were evaluated using the paired *t* test. The responder criteria are defined as a twofold increase of anti-rabies antibody titers for both the primary and secondary response. A SI ≥ 3.0 was considered as evidence of antigen-induced lymphocyte proliferation. Lymphocyte proliferative responses after first and booster vaccination were compared by the paired *t* test. The $p < 0.05$ was considered to be statistically significant.

Results

Study population

Study subjects included 18 healthy volunteers, 9 women and 9 men. The average age was 31 (range 19 to 49 years). All healthy adults had a normal serum concentration of total IgG, IgA and IgM, and normal leukocyte counts and differentials (data not shown). Normal proliferation was found in all healthy adults following PHA and αCD3 stimulation of PBMC (data not shown). During the trial two persons mentioned muscular tenderness at the injection site after the first vaccination. Two

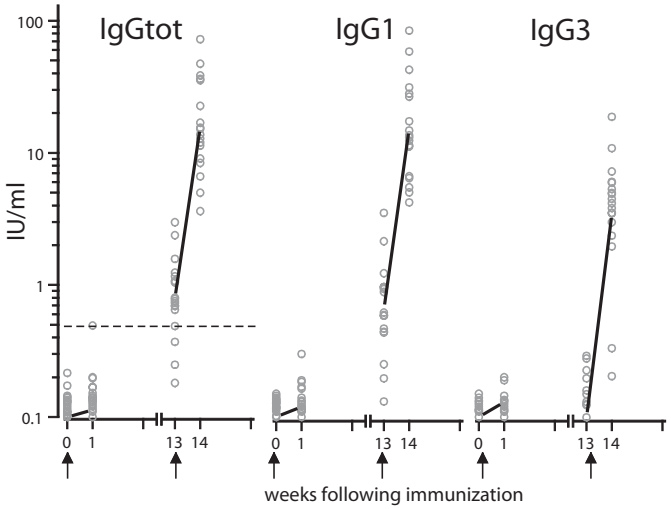

Figure 2. Concentration of total IgG, IgG1 and IgG3 anti-rabies one week following primary (week 0) and booster (week 13) immunization (*arrows*).

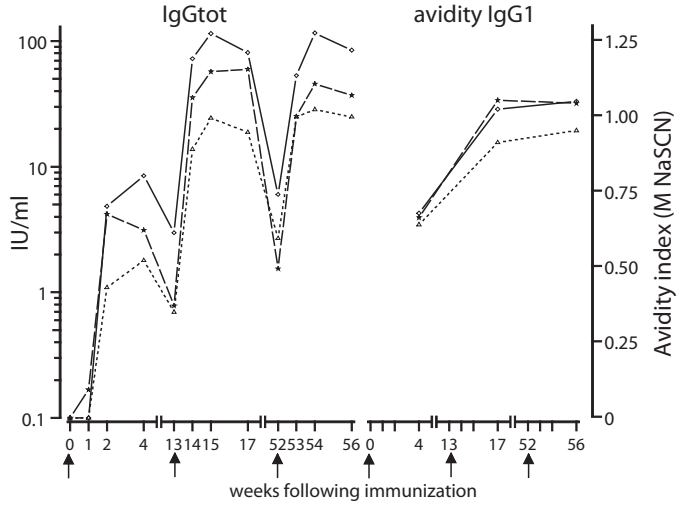

Figure 3. IgG anti-rabies (IU/mL) and avidity index (AI) of IgG1 following three immunizations in three healthy adults. *Arrows* indicate week of immunization

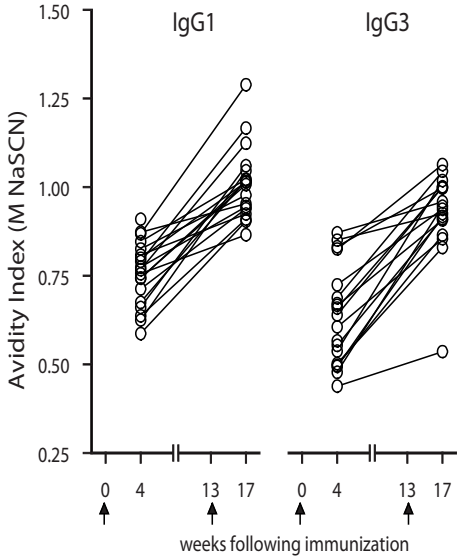

Figure 4. Relative avidity index (AI) of IgG1 and IgG3 anti-rabies in 18 healthy adults, 4 weeks following primary vaccination at time-point 0 and booster vaccination at week 13, respectively. *Arrows* indicate week of immunization.

other persons had an evanescent generalized rash, occurring one week after the first and second vaccination, respectively. After the second vaccination one person reported headache and malaise. Five patients diagnosed with a combined B- and T-cellular immunodeficiency, of which three siblings, included three girls and two boys, average age of 8.5 (range 4-13 years). No adverse effects were reported in the patients after both vaccinations.

Humoral response in healthy volunteers: antibody concentration

All 18 healthy individuals analyzed mounted an anti-rabies antibody response in the IgG, *i.e.* IgG1 and IgG3 subclasses, IgM and IgA isotypes after a primary and after a booster immunization. The GMTs of anti-rabies antibody concentrations and the kinetics of the response are shown in Figure 1, and GMTs and the 95% CI are summarized in Table I. The IgG2 and IgG4 anti-rabies antibodies were not detectable following primary or booster vaccination. Comparison between males and females revealed no significant difference in antibody responses. The GMTs at each time-point could be considered normally distributed, according to the Kolmogorov-Smirnov one-sample test (data not shown). After primary immunization, a significant rise of GMT was observed at 1 week for IgM and at 2 weeks for IgG (IgG1 and IgG3) and IgA. A mean 31-fold increase (range 3-154) of IgG antibody titer was reached four weeks after the first vaccination. One person did not reach the protective IgG antibody titer of 0.5 IU/mL (13) at this time-point. The kinetics of the response of IgM and IgA differed from that of IgG in that the highest GMTs were obtained at 2 weeks after

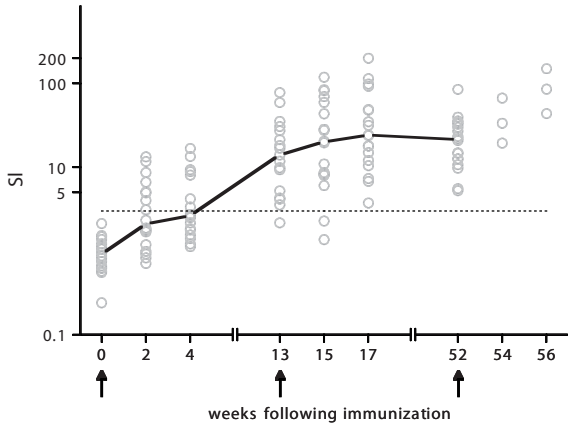

Figure 5. Stimulation index (SI) of rabies-induced proliferation in cultures of PBMC from 18 healthy adults following rabies immunizations. *Arrows* indicate week of immunization. Three individuals received a third immunization at week 52.

primary immunization, compared with 4 weeks for IgG. After booster immunization the antibody response clearly showed characteristics of a secondary response, *i.e.*, a rapid and strong increase of the IgG antibody response, an isotype switch from IgM to IgG and, to a lesser extent, to IgA (Figure 1). Overall, after booster vaccination the anti-rabies IgG response was significantly faster (evaluated one week after vaccination, paired *t* test, $p<0.001$) and stronger (every time-point, paired *t* test, $p<0.001$) compared to the response after the primary vaccination.

The highest IgG (IgG1 and IgG3) anti-rabies GMTs were detected 2 weeks following the booster immunization, compared to 4 weeks after the primary immunization. At 1 year following primary immunization and 9 months after secondary immunization, GMTs of IgG anti-rabies had declined (Table I). Nevertheless, the seroconversion level (>0.5 IU/mL) was still achieved in all 18 adults at this time-point. The optimal parameter for discriminating between the primary and the secondary humoral immune response for the whole study group of healthy adults turned out to be the concentration of total IgG, IgG1 and IgG3, anti-rabies one week after the respective vaccinations. At that time-point no overlap in data between primary and secondary vaccination was observed (Figure 2). The GMTs of IgG anti-rabies antibodies and the kinetics of the response following a third immunization in three individuals were comparable to results found after the second vaccination (Figure 3). After the third vaccination, an evident but low IgG4 titer was measured in one person. Overall, IgG1 is the major IgG subclass in which antibodies were formed (mean 83% of total IgG, range 54-98%) after primary and secondary rabies immunization. We did not find any individual, vaccination- or time- dependent effect on the proportion of IgG1 and IgG3 anti-rabies antibodies.

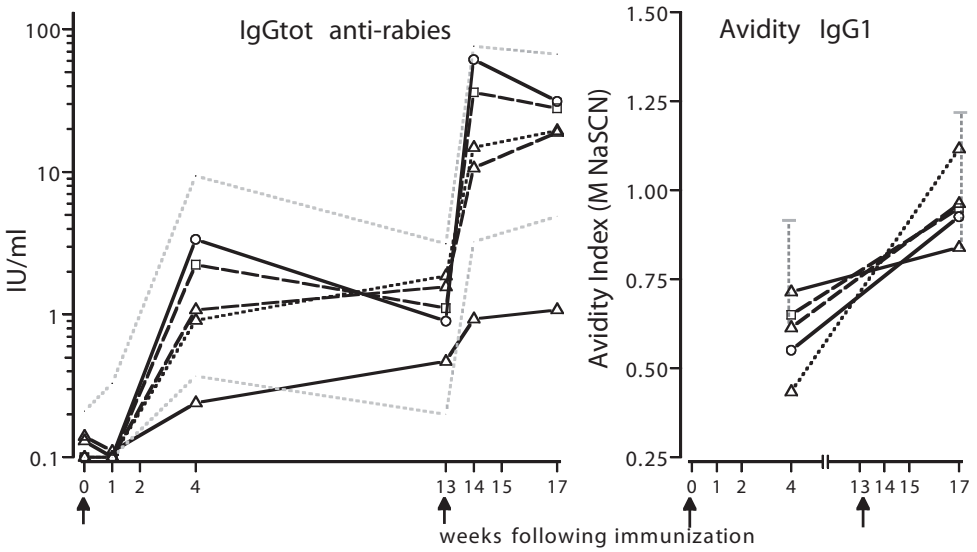

Figure 6. IgG anti-rabies concentration (IU/mL) and relative avidity index (AI) of IgG1 anti-rabies in five patients with a of combined immunodeficiency following primary (at week 0) and booster (at week 13) vaccination (*arrows*). Area between *dashed lines* (left figure) represents the 95% confidence interval of IgG anti-rabies concentration in the 18 healthy adult individuals vaccinated according to the same protocol.

Table II. Rabies Induced I*n Vitro* Proliferative Response

Week	Mean SI	95% CI	Significance
0	0.9	0.4, 1.5	
2	4.0	0.8, 12.6	**
4	4.8	1.2, 15.4	**
13	20.7	2.7, 70.6	**
15	35.7	3.8, 105.1	***/NS versus week 13
17	45.0	5.0, 163.9	***/NS versus week 13
52	24.2	5.4, 65.8	***/NS versus week 13

Note. Stimulation index (SI) and 95 % confidence interval (CI) of rabies induced *in vitro* proliferative response of PBMC at the indicated time-points after primary (week 0) and secondary (week 13) immunization with rabies vaccine.

Statistics: Paired *t* test (* compared to week 0): NS not significant, ** p< 0.01, *** p < 0.001.

Humoral response in healthy volunteers: antibody avidity

Because IgG1 and IgG3 anti-rabies antibodies were quantitatively the major IgG subclasses formed, antibody avidity was measured within these subclasses. The relative avidity index (AI) was 0.8 for IgG1 and 0.65 for IgG3 at 4 weeks after the primary immunization (Figure 4). A significant increase was measured at 4 weeks after booster immunization, *i.e.* from mean 0.8 to 1.0 for IgG1 (paired *t*

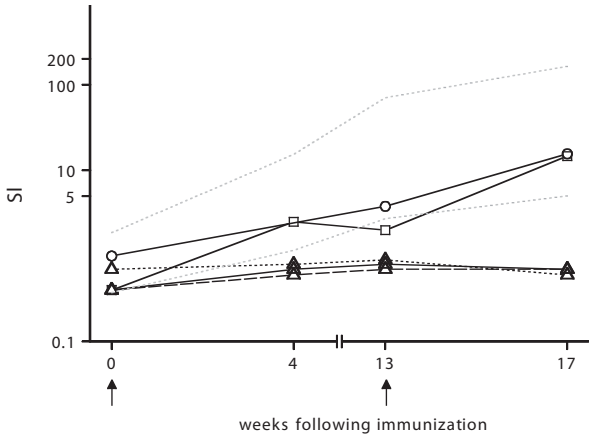

Figure 7. Stimulation index (SI) of rabies-induced proliferation in cultures of PBMC in five patients with a combined immunodeficiency following rabies immunizations. Area between *dashed lines* represents the 95% confidence interval of the proliferative response in cultures of PBMC from the 18 healthy adult individuals. *Arrows* indicate week of immunization.

test IgG1, p<0.001) and from 0.65 to 0.90 for IgG3 (paired *t* test IgG3, p<0.001) (Figure 4). Also the Shannon index, measure of avidity heterogeneity (mean after primary immunization: 11.0; mean after secondary immunization: 13.1), and the Luxton index, representing the contribution of high affinity antibodies (mean after primary immunization: 40; mean after secondary immunization: 109) for IgG1 anti-rabies showed a significant increase after booster vaccination (data not shown). For IgG3 similar changes of these indices were found (data not shown). The AI of IgG1 anti-rabies antibodies after a third immunization of three individuals was comparable to that after the second vaccination (Figure 3).

Lymphocyte proliferative response in healthy volunteers

PBMC from 18 healthy adults, following primary and booster HDCV rabies vaccination, were stimulated *in vitro* with DKCV. Proliferation was determined by ^{3}H-thymidine incorporation and SI of single individuals, and mean SI and 95% confidence intervals (CI) are shown in Figure 5 and Table II, respectively. At 4 weeks after primary immunization 7 of 18 healthy persons showed a SI ≥ 3 (mean SI whole group 4.8, range 1.0-17), and all individuals reached a SI ≥ 3 (mean SI 45, range 3.5-200) at 4 weeks after secondary immunization. The highest SI after primary immunization was measured at 13 weeks post vaccination. All individuals showed a significant increase of the proliferative response after booster immunization (paired *t* test, 4 weeks after primary immunization versus 4 weeks after secondary immunization, p=0.004). Four weeks post booster immunization, uptake of ^{3}H-thymidine after *in vitro* DKCV stimulation of PBMC ranged from 1680 to 96460 cpm (median 48320), versus medium control ranged from 60 to 5770 cpm (median 1000). Following a third rabies vaccination in 3 healthy individuals a further increase of SI was observed.

Humoral and cellular response in patients with a combined immunodeficiency

Four of the 5 patients suffering from a combined immunodeficiency mounted a quantitatively and qualitatively normal primary and secondary IgG anti-rabies response (Figure 6). In one patient only low IgA anti-rabies titers were found (data not shown). In another patient IgG as well as IgM and IgA (data not shown) anti-rabies antibody levels were lower after vaccinations in comparison to the healthy controls. However, also in this patient normal avidity maturation was observed after secondary immunization. This patient, together with her two siblings, showed a different kinetics of antibody formation compared to the healthy adult controls (Figure 6), *i.e.*, highest antibody titers were detected 13 weeks after the primary immunization instead of at 4 weeks. In 3 out of 5 patients a decreased *in vitro* proliferative response to rabies was found (Figure 7). Two of these three patients showed a normal humoral immune response upon rabies vaccination.

Discussion

This study was performed to characterize the humoral and cellular immune response following a primary and a booster vaccination with the T-cell dependent neoantigen rabies in healthy individuals. Therefore, we analyzed several parameters of the anti-rabies immune response; *i.e.,* the Ig isotype and IgG subclass distribution, and the avidity maturation of specific antibodies, and the *in vitro* T-cell proliferative response to the neoantigen. Such characteristics would provide a reference for the use of this neoantigen in the assessment of the immunological competence of patients suspected of primary or secondary immunodeficiencies. Specific antibodies to rabies antigen were formed in IgM, IgA, IgG1 and IgG3. We confirmed that healthy individuals are already primed after a single rabies vaccination, independently of the achievement of seroconversion (anti-rabies IgG ≥ 0.5 IU/ml) as formulated by the WHO (13). All 18 healthy individuals showed characteristics of a memory response after the booster vaccination at 3 months after the primary vaccination, *i.e.,* a rapid increase of IgG antibody levels, an isotype switch from IgM to IgG and IgA, and an increase of the antibody avidity. All healthy individuals showed a significant *in vitro* proliferative response (defined as SI ≥ 3) following primary (except for one person) and booster rabies immunization Although discrimination between a primary and a secondary response to the T-cell dependent neoantigen rabies is possible for every healthy person on an individual basis, for the whole group there is some overlap in results at the different time-points post vaccination. The most discriminating parameter appeared to be the concentration of IgG anti-rabies antibodies 1 week after HDCV vaccination. Quantification of rabies-specific IgM using whole virus was hampered by non-specific binding that results in high preimmune values. Although a number of different ELISA methods and modifications were evaluated to minimize the background values, the best results were obtained with a commercial kit containing a purified glycoprotein as coat. Nonspecific binding of IgM to rabies and also to other antigens has been described before and may be caused by a cross-reaction due to molecular mimicry or shared epitopes with other micro-organisms (22, 23). Molecular mimicry between rabies virus glycoprotein and the HIV glycoprotein 120 has been confirmed by the detection of circulating

cross-reacting antibodies (24). Circulating IgM rheumatoid factor (IgM anti-human IgG) is also a well-known problem in IgM serological testing (25). Nonetheless, removal of IgG from our sera gave no improvement in background values.

Both in healthy adults and in the patients suspected of a combined immunodeficiency, IgG1 was the predominant IgG subclass in the response to rabies, followed by IgG3. IgG2 could not be detected, and IgG4 anti-rabies was formed in one of three persons after the third vaccination. In contrast to reports on other neoantigens, the developmental sequence of the appearance of antibodies in the different IgG subclasses, *i.e.*, first IgG3 than IgG1, after successive immunizations was not observed for rabies (26). A response in IgG4, considered as a "memory" IgG subclass, may become more pronounced after longer follow-up and more frequent immunizations. Sofar, little is known about Ig (sub)class distribution and avidity maturation following rabies vaccination (27). To our knowledge, this is the first study clearly showing avidity maturation of rabies antibodies following a single booster immunization. In other studies, IgG anti-rabies antibodies are only determined following multiple (at least three) immunizations, and different methods for detection of anti-rabies antibodies are used (28-31). Despite these shortcomings for comparison of data, following a series of HDCV rabies vaccinations at day 0, 7 and 28, neutralizing antibodies were formed as early as 7 days of vaccination. At 2-4 weeks after a third vaccination highest IgG antibody titers were found and titers ≥ 0.5 IU/mL could be identified for at least 2-14 years (29, 30, 32, 33). Two weeks following completion of pre-exposure schedules the anti-rabies antibodies ranged from 1.3 to 140 IU/mL (12, 30), which is comparable to the responses determined after two vaccinations separated by 3 months. A rapid decline in antibody titers 1 year following immunization and no further increase of antibody titers following a third booster immunization have been reported by others as well (12). No differences in antibody responses have been found between healthy children and adults (34, 35).

The cellular immune response following rabies vaccination has previously been studied to determine the role of cell-mediated immunity in the protection against, and the immunopathogenesis of, rabies. The capability of rabies human vaccines to induce *in vitro* lymphocyte proliferation has been reported by several groups, but again only following multiple vaccinations in pre- and post-exposure vaccination regimens (2, 36, 37). Our study is the first to demonstrate the changes of T-cell proliferative responses following a primary rabies vaccination and a single booster immunization at several months after priming. The magnitude of the *in vitro* proliferative response is adequate to differentiate between a primary and secondary immune response in healthy individuals. The cpm and SI values 3 months after the primary vaccination were comparable, and even higher compared to results obtained by others after a series of at least 3 vaccinations within the restricted period of 1 month (29, 36, 38). In contrast to our data, others (39) detected high preimmunization proliferation in some healthy individuals, ascribed to a natural cellular reaction to the internal common N protein of the virus or by superantigen-like properties of rabies virus nucleocapsid (40).

Rabies vaccine is mainly used for disease prevention, only two studies have been published investigating rabies as a test (neo)antigen. Ghaffari *et al.* (39) have investigated T-cell proliferation and IgG antibody response prior to and 2 weeks after three subsequent vaccinations in 14 healthy volunteers. However, they did not study the characteristic differences following a primary and a

booster vaccination, a very relevant aspect in studying the development of the immune response. Korver *et al.* has also used rabies vaccine as a neoantigen in the early eighties (6). Dose response effects of primary immunization with a variable dosage of DKCV rabies vaccine in healthy volunteers and in patients treated with hemodialysis or continuous ambulatory peritoneal dialysis (CAPD) were studied. Higher doses of rabies vaccine were required to induce a proper IgM and IgG response in the immunocompromised patients. In our study of five patients with a combined immunodeficiency, the humoral immune response to rabies vaccination showed that there was no evidence of an intrinsic B-cell defect in four of them. Despite reduced numbers of circulating B cells, and a severely decreased immune response following vaccination with the T-cell dependent recall antigens tetanus toxoid and conjugated *Haemophilus influenzae* type b polysaccharide, these patients proved to be able to produce antibodies with a normal IgM to IgG isotype switch and avidity maturation after booster immunization with the T-cell dependent neoantigen rabies. In one patient a low level of anti-rabies IgG was found, although the relative avidity of the formed IgG1 was normal. A decreased, or low normal *in vitro* proliferative response to rabies was detected in all patients. According to standard criteria (1) they could be suspected of common variable immunodeficiency (CVID). CVID is a heterogeneous group of primary immunodeficiency diseases, in which B-cell and/or T-cell function may be affected, characterized by hypogammaglobulinemia and defective antibody formation, resulting in recurrent bacterial infections (41, 42). Given our results, CVID as diagnosis is less plausible. The failure to elicit an *in vitro* proliferative response to a T-cell dependent neoantigen in our patients could be due to a disturbed antigen presentation (43, 44) or a defect originating within the T-cells. A possible explanation for the different kinetics of the humoral anti-rabies immune response in the three sibling patients, *i.e.,* a delay of the peak of specific antibodies in comparison to healthy controls may point to a dysfunction in APC, resulting in reduced antigen clearance (44). Immunomodulatory effects induced by IVIG therapy may also influence the kinetics of the anti-rabies antibody response (45, 46). Taking these patients as an example, we illustrate that investigation of the humoral and cellular immune response to rabies, as a T-cell dependent neoantigen, may help to further differentiate between underlying defects in patients suspected of a combined immunodeficiency.

In the study group of healthy adults, five out 18 individuals mentioned relatively mild side effects of the intramuscular vaccination, including two systemic reactions consisting of an urticarial rash. In the group of five patients no adverse effects were reported.

Conclusion

We conclude that rabies vaccine is a safe and effective antigen for measurement of both B- and T-cell immune responses to a neoantigen in man. The results obtained in this study provide a frame of reference for the interpretation of specific immune responses to the T-cell dependent neoantigen in patients suspected of a primary or secondary immunodeficiency. Currently, antigen-specific

immune reconstitution in HIV patients following HAART therapy and in several autoimmune patients following autologous stem cell transplantation is being investigated using rabies as antigen.

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