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Detection of mumps virus-specific memory B cells by transfer of peripheral blood mononuclear cells into immune-deficient mice

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Summary

Waning immunity to mumps after one or two doses of the measles, mumps and rubella (MMR) vaccine has been described. Using a human peripheral blood lymphocyte (PBL)–severe combined immunodeficiency (SCID) mouse model, MMR vaccine recipients with undetectable and high antibody titres against mumps were compared for the presence of circulating mumps-specific memory B cells. Peripheral blood mononuclear cells (PBMC) from six donors (three subjects with undetectable and three with high antibody titres against mumps) were injected into the spleens of non-obese diabetic (NOD)-SCID mice (three mice per subject). Mice were pretreated with TMβ1 and total body irradiation to improve engraftment. *In vivo* production of human antibodies against mumps was evaluated in mouse plasma on days 7, 10 and 13 with a commercial enzyme-linked immunosorbent assay (ELISA), functional reduction neutralization test. Three donors had mumps antibody titres below the detection limit (titre < 230) and three had high antibody titres (range 5700–7300). None of the mice injected with PBMC from subjects with undetectable antibody titres showed detectable human antibody titres, despite the presence of cell-mediated immunity in two of the three donors. Seven out of nine mice injected with PBMC from subjects with high antibody titres acquired detectable antibody titres for mumps in their plasma. PBMC from vaccinees without detectable serum antibodies against mumps virus were unable to induce secretion of anti-mumps antibodies in the blood of recipient mice, whereas PBMC from vaccinees with high antibody titres were able to do so. This observation suggests that the frequency of mumps-specific memory B cells is very low in vaccinees with undetectable antibody titres. These individuals may therefore be at risk of developing mumps disease upon encounter with wild-type virus.

Keywords: B cell; memory; mumps; vaccination; viral

Introduction

Prior to vaccination, mumps was a viral infection that caused high morbidity, such as meningitis, orchitis and deafness in different age groups and populations.[1] Vaccines against mumps have been available for decades and their systematic use has significantly reduced the incidence and associated complications of mumps. Nevertheless, the resurgence of mumps in recent outbreaks in several Western countries has raised awareness of mumps and its prevention. Despite initial success in controlling this infection through vaccination, elimination of mumps seems hard to achieve.[1,2]

The humoral immune response induced by mumps vaccines has been explored in several studies. Antibody titres have been measured either a few weeks or many years after immunization. Prelicensure studies of mumps vaccines in seronegative children showed high seroconversion rates (approximately 90–95%) after immunization.[3] In contrast, several post-marketing studies have shown that susceptibility to mumps infection increases with time after one or two vaccine doses, which may lead to outbreaks of mumps, in various populations.[4–6] In two recent longitudinal studies of individuals who received two measles, mumps and rubella (MMR) doses, the second of which was given 5–15 years before the last blood

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sample was taken, a decline in geometric mean titre over time and a decrease in the proportion of subjects with detectable circulating antibodies were observed.[7,8]

In order to take adequate measures against infection, it is essential to know whether a vaccinated person becomes susceptible to infection with mumps when mumps-specific antibodies reach a critically low level or become undetectable. For numerous infections, the levels of antibodies produced by long-lived plasma cells are the best and often the only markers for estimating vaccine efficacy, although vaccine-induced protection can also be mediated by other effectors of the adaptive immune system, such as T helper (Th) cells or cytotoxic T lymphocytes (CTLs). In addition to immediate protection, our immune system also develops immune memory in the form of memory B and T cells. To assess the long-term protection induced by vaccination, it may be useful to evaluate the presence and number of circulating memory B cells, especially when antibodies have reached low or undetectable levels at some time-point after vaccination.[9]

Several techniques can be used to detect and enumerate memory B cells, such as limiting dilution analysis, the B-cell enzyme-linked immunosorbent spot-forming cell assay (ELIspot) and multicolour flow cytometry.[9–12] Drawing on our extensive experience with a non-obese diabetic (NOD)–severe combined immunodeficiency (SCID) mouse model for the detection of hepatitis C virus-specific memory B cells, we transferred peripheral mononuclear blood cells (PBMC) into conditioned NOD-SCID mice to reveal the presence of mumps-specific memory B cells in the circulation of vaccine recipients [the human peripheral blood lymphocyte (PBL)–SCID mouse (Hu-PBL-SCID) model]. Through analysis of the plasma of chimeric mice, this model allows the detection of antibody production by circulating memory B cells from subjects in whose serum such antibodies are no longer detectable.[13] This model has several advantages: (i) there is considerable expansion of the human B-cell population in the mouse spleen; (ii) the model does not require antigen for detection of antigen-specific memory B cells, as is the case with *in vitro* tests; (iii) as this is an *in vivo* test system, not only the presence but also the functionality of the memory B cells is measured. The disadvantage of this model is that it is qualitative and does not allow the enumeration of antigen-specific memory B cells.

Until now, mumps-specific memory B cells have been analysed using flow cytometry in persons with positive antibody titres as a result of previous mumps disease.[14] To extend our knowledge of the presence of mumps-specific circulating memory B cells in immunized individuals, we evaluated the production of mumps-specific antibodies by the PBMC of vaccinees with either undetectable or high antibody levels in the Hu-PBL-SCID model. To our knowledge, this is the first study examining the presence of mumps-specific memory B cells in immunized individuals.

Materials and methods

Population and serological assays

Six subjects (all women; mean age 19·8 years) were selected from a cohort of university students in whom humoral and cellular immune responses to mumps had been measured previously.[15,16]

Three subjects were chosen because their antibody levels for mumps were below the lower limit of detection of the assay (titre < 230). For comparison, three subjects were selected with high antibody titres (5700, 6000 and 7300). All subjects had documentation of at least one dose of mumps-containing vaccine, were free of acute or chronic ailments and did not have an immune deficiency induced by disease or medication. Immunization dates were double-checked using school health records.

In the low-antibody group, two subjects were given two MMR vaccines and one received only one dose of MMR vaccine at 14 months of age. In the high-antibody group, two subjects received two MMR vaccine doses and one subject received a single dose at the age of 14 years (Table 1). The two subgroups were comparable with respect to age at blood sampling (19·7 years versus 19·8 years). However, the interval between the last immunization and the moment of blood sampling differed significantly (11·6 years for the low-antibody group versus 5·5 years for the high-antibody group) (Table 1).

Peripheral blood mononuclear cells for transplantation

PBMC from each participant were isolated from 50 ml of heparinized venous blood by isopycnic density gradient centrifugation. After two washes in Hank's balanced salt solution (HBSS), the cells were re-suspended in fetal bovine serum (FBS) (BioWittaker, Walkersville, MD)

Table 1. Details of the measles, mumps and rubella (MMR) vaccination of the subjects

Donor number	Number of vaccines received	Age at blood sampling	Time interval since last vaccination
Low-antibody group			
68	2 MMR	18 years 10 months	7 years
110	1 MMR	20 years 11 months	19 years 5 months
157	2 MMR	19 years 9 months	7 years 9 months
High-antibody group			
49	2 MMR	18 years 6 months	6 years 6 months
90	2 MMR	19 years 6 months	4 years 7 months
97	1 MMR	19 years 8 months	5 years 6 months

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supplemented with 10% dimethyl sulphoxide (DMSO) (Sigma, St Louis, MO) and cryopreserved in liquid nitrogen until use. PBMC were thawed and washed three times with HBSS and finally re-suspended in phosphate-buffered saline (PBS) (± 10^7 in 50 μl). After thawing and washing, the viability of the PBMC exceeded 95% in five samples and was 88% in the sixth sample, as determined by propidium iodide exclusion in flow cytometric analysis.

Lymphoproliferation assay

Cell-mediated immune responses to mumps vaccine viruses used as antigens were assessed using an *in vitro* [^{3}H]thymidine incorporation assay as previously described.[16] After thawing, PBMC were washed three times with HBSS and re-suspended in complete RPMI-1640 (Gibco, Invitrogen, Carlsbad, CA) containing 10% FBS. PBMC were stimulated with active monovalent mumps vaccine (Mumpsvax®; Merck, Whitehouse Station, NJ) at a multiplicity of infection (MOI) of 25×10^5. The vaccines were reconstituted in 1 ml of complete RPMI-1640. Cultures containing media without antigen served as a negative control (blank) and cultures re-stimulated with either tetanus toxoid (TT) or varicella zoster virus (VZV) lysate served as positive controls. Phytohaemagglutinin (PHA) at 4 μg/ml (Sigma) was used to demonstrate the capacity of the PBMC to proliferate. After 2 days (for PHA) and 5 days (for mumps, TT and VZV) 0·5 μCi [^{3}H]thymidine (Amersham, GE Healthcare, Uppsala, Sweden) was added to the cultures and [^{3}H]thymidine incorporated during the final 16 hr of the culture was quantified by liquid scintillation counting. The geometric mean (geomean) of the counts per minute (c.p.m.) for unstimulated PBMC and PBMC stimulated with measles and mumps vaccine virus, TT and VZV lysate was calculated.[16] The stimulation index (SI) was the ratio of geomean c.p.m. in antigen-stimulated and unstimulated control wells. Responses were considered positive when the SI was ≥ 3.

The Hu-PBL-SCID model

The Hu-PBL-SCID model has been fully described previously.[13] In brief, NOD-SCID mice (NOD/LtSz-Prkdc^{scid}/Prkdc^{scid}) were bred under sterile conditions and fed *ad libitum* with autoclaved food and water without the addition of prophylactic antibiotics. The NOD/LtSz-Prkdc^{scid}/Prkdc^{scid} mouse is known to have reduced natural killer (NK) activity, macrophage function and serum haemolytic complement activity. The NOD-SCID strain was free of Emv30, an endogenous murine ecotropic retrovirus responsible for induction of lethal thymomas. Mice used were between 8 and 12 weeks of age.

The day before the transfer of the PBMC (day –1), mice were conditioned with a sublethal dose of total body irradiation (3 Gy) and a single intraperitoneal injection of 1 mg of purified TMβ1 in 500 μl of PBS. TMβ1 is a rat monoclonal antibody directed against the murine interleukin (IL)-2 receptor β-chain that was produced in the laboratory of the Center for Vaccinology as described[13,17]. Administration of TMβ1 induces *in vivo* depletion of mouse NK cells.[13,17] The total body irradiation facilitates the engraftment of the human leucocytes in the spleen.[13]

PBMC (10^7 in 50 μl per animal) were injected directly into the spleen of the recipient animal. For this procedure, mice were anesthetized and a subcostal incision into the skin was made followed by incisions into the abdominal wall and the peritoneum. The spleen was carefully exposed and the 50-μl cell suspension (10^7 PBMC) was injected with great care. The spleen was then repositioned in the abdominal cavity, and the abdominal wall and skin were sutured layer by layer. PBMC from each of the six participants were injected into three mice. On days 7, 10 and 13 (if the animal was still alive), blood was drawn into ethylenediaminetetraacetic acid (EDTA)-coated capillaries from the retro-orbital plexus of mice under anesthesia. Plasma was prepared and stored at −20° until analysis.

Quantification of anti-mumps antibody levels in human sera and mouse plasma

Anti-mumps antibody levels in human sera and mouse plasma were measured using a mumps virus-specific immunoglobulin G (IgG) enzyme immunoassay (EIA) (Siemens, previously Dade Behring, Erlangen, Germany), which uses a whole mumps virus lysate of the Enders strain VR-106 as an antigen. Sera were diluted according to the manufacturer's instructions. Mumps-specific antibodies are expressed as titres. According to the manufacturer's instructions, subjects were considered seronegative if the antibody titre was < 230.

Detection of mumps-specific neutralizing antibodies

The human and mouse sera were tested using the mumps focus reduction neutralization test (FRNT) protocol previously described by Vaidya *et al.*, with minor modifications.[18] In brief, twofold serum dilutions (1/2 to 1/256) were performed in growth medium supplemented with 15% FBS. To these serum dilutions, an equal volume (40 μl) of challenge mumps virus (titrated to give 30–45 foci per well) was added, and plates were incubated at 37° in 5% CO_2 for 2 hr. After incubation, 20 μl of each reaction mixture was inoculated in triplicate into wells of a 96-well plate containing a 24-hr-old preformed Vero cell monolayer, which was incubated for 4 hr in a 37°/5% CO_2 incubator. Virus controls were prepared without

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serum samples. The virus/serum mix was then removed and each monolayer overlaid with 150 μl of 1% Avicel (FMC Biopolymer, Philadelphia, PA). The plates were incubated for 2 days, and were developed as previously described.[18] After development, the foci were counted using a CTL Biospot analyser (Immunospot, Shaker Heights, OH) and 50% focus reduction neutralization titres were calculated using the Kärber formula. A mumps neutralizing antibody titre > 4 is considered positive.

Ethical considerations

The human part of this study was approved by the Ethics Committee of the University Hospitals of the Katholieke Universiteit Leuven and carried out according to the latest Declaration of Helsinki. The experiments in NOD-SCID mice were approved by the Animal Ethics Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Results

Three NOD-SCID mice were injected with 10^7 PBMC from each of the six participants. All 18 mice progressively showed clinical signs of graft-versus-host disease, which indicated that the graft take was successful. One animal died before blood was taken on day 10, and 12 animals died between days 10 and 13.

Table 2 summarizes the results of this study. The individual donor numbers are shown in the left column. Column 2 shows the anti-mumps antibody levels of the individual donors, column 3 the results of the FRNT, and column 4 the results of T-cell lymphoproliferation for mumps. Three donors (numbers 68, 110 and 157) had anti-mumps IgG levels below 230 titres and three (49, 90 and 97) had high antibody titres. Of the donors with low IgG levels measured by ELISA, only one (157) had a positive neutralizing antibody titre. Two low-titre donors (68 and 110) had mumps-specific lymphoproliferative responses with SI > 3, indicating that previous vaccination had induced a persistent T-cell immune response. All high-titre donors displayed mumps-specific neutralizing antibodies and T-cell responses with SI > 3.

Further to the right, the table shows antibody levels induced in three mice (numbers 1, 2 and 3) injected with PBMC per donor, for each of the six donors. Mumps-specific IgG levels measured on day 7 (column 6), day 10 (column 7) and where available on day 13 (column 8) are shown. ELISA titres are shown first, and the neutralizing antibody titres are shown in brackets. The anti-

Table 2. Donor anti-mumps antibody response [enzyme-linked immunosorbent assay (ELISA) and focus reduction neutralization test (FRNT)] and cell-mediated immunity to mumps and *in vivo* evoked mumps antibody response (ELISA and FRNT) in the human peripheral blood lymphocyte (PBL)–severe combined immunodeficiency (SCID) mouse (Hu-PBL-SCID) model

Donor number	Donor Antibody response (ELISA and FPRN) and CMI			Mouse number	Hu-PBL-SCID ELISA antibody response (titre)* FRNT (neutralizing antibody titre)		
	IgG (titre)	FPRN (titre)	CMI (SI)		Day 7	Day 10	Day 13
68	< 230	< 4	**4·6**	1	< 230 (< 4)	< 230 (< 4)	< 230 (< 4)
				2	< 230 (< 4)	< 230 (< 4)	
				3	< 230 (< 4)	< 230 (< 4)	< 230 (< 4)
110	< 230	< 4	**5·0**	1	< 230 (< 4)	< 230 (< 4)	< 230 (< 4)
				2	< 230 (< 4)	< 230 (< 4)	
				3	< 230 (< 4)	< 230 (< 4)	
157	< 230	**12·8**	0·8	1	< 230 (< 4)	-	
				2	< 230 (< 4)	< 230 (< 4)	
				3	< 230 (< 4)	< 230 (< 4)	
49	**7300**	**116·1**	**6·7**	1	< 230 (< 4)	< 230 **(5·7)**	
				2	< 230 (< 4)	**950** (< 4)	
				3	< 230 (< 4)	< 230 (< 4)	**360** (< 4)
90	**6000**	**60·5**	**4·3**	1	< 230 (< 4)	**1100 (17·8)**	
				2	< 230 (< 4)	< 230 **(5·9)**	**250 (63·9)**
				3	< 230 (< 4)	**430** (< 4)	
97	**5700**	**38·6**	**9·6**	1	< 230 (< 4)	**320** (< 4)	
				2	< 230 (< 4)	**1100** (< 4)	
				3	< 230 (< 4)	< 230 (< 4)	

*Blood was only drawn from mice who survived until day 13.
Positive tests (ELISA, FRNT and CMI) are shown in bold.
CMI, cell-mediated immunity; IgG, immunoglobulin G; SI, stimulation index.

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mumps IgG levels in the serum from donors 68, 110 and 157 were below the threshold of 230. Injection of PBMC from these donors into NOD-SCID mice did not induce detectable anti-mumps IgG levels in the recipient mice. Transfer of PBMC isolated from donors with high-titre anti-mumps IgG in circulation induced anti-mumps IgG detectable as of day 10, in five of nine recipient mice. Two animals receiving PBMC from donor 49 (mouse 1) and donor 97 (mouse 3) did not respond. In two additional mice, the titre rose above the threshold on day 13. Overall, seven of nine mice injected with PBMC from donors with high-titre circulating anti-mumps IgG displayed detectable mumps-specific IgG production in their plasma.

When neutralizing antibodies were tested in the sera of transplanted mice, three of these mice showed *in vitro* mumps-neutralizing acitivity in four different serum samples. Overall, neutralizing antibodies followed the same trend as antibodies measured by ELISA, as no neutralizing antibodies could be detected in the 'low-antibody group'. However, not all mice transplanted with PBMC from 'high-antibody donors' and in which antibodies were detected by ELISA displayed neutralizing activity.

Discussion

We show here for the first time that vaccine-induced memory B cells capable of secreting mumps virus-specific IgG can be detected by intrasplenic transfer of human PBMC into the spleens of NOD-SCID mice. A strong correlation was noted between the presence or absence of virus-specific IgG in the sera of cell donors and the capacity or inability of the respective PBMC to secrete ELISA-detectable levels of virus-specific IgG in the chimeric mouse plasma.

In this study, antibodies were measured with a commercial ELISA and with a novel FRNT as developed by Vaidya *et al.*[18] The results from the FRNT are different from those of the ELISA. With the plaque reduction neutralization test, lower levels of antibodies can be detected because the serum requires less dilution than for ELISAs.[19] Although there was not a perfect match between the ELISA and FRNT results, in this study neutralizing antibodies were only detected in mice who were injected with PBMC from high-titre donors. The discrepancy between the ELISA and FRNT results may be attributable to differences in the analytical sensitivities of the tests. Alternatively, B cells secreting antibodies that can be detected by ELISA may be more abundant than B cells producing neutralizing antibodies.

These data suggest that, in vaccinees without detectable serum antibodies against mumps viruses, no or extremely low numbers of memory B cells against these viruses are circulating in the venous blood. In vaccine recipients who have retained high-titre anti-mumps IgG in the circulation, memory B cells that can be stimulated to differentiate into active plasma cells producing anti-mumps antibodies remain in the circulation at frequencies that allow the production of detectable virus-specific IgG responses *in vivo* in the transplanted mice.

In contrast to other methods used for the detection of memory B cells, such as ELIspot or flow cytometric analyses, the transfer of PBMC into NOD-SCID mice does not provide quantitative information on the frequencies of antigen-specific B cells. However, the intrasplenic injection of human PBMC into NOD-SCID mice turned out to be a very sensitive method for revealing the presence of antigen-specific memory B cells in the circulating memory B-cell pool.[13] Nevertheless, correlations between antibody levels and the number of memory B cells cannot be made using this method. Amanna *et al.* demonstrated a correlation between memory B-cell numbers and antibody titre for antigens from acute infections (such as mumps, measles and rubella), but this was not the case for antigens from other acute infections (such as vaccinia), for protein vaccines (diphtheria and tetanus), or for chronic infections (Epstein–Barr virus and VZV).[14]

The number of individuals participating in this study was very small. Conclusions should therefore be drawn with care and extrapolations made cautiously. However, the consistency of the results obtained in three mice injected with PBMC from each participant is reassuring.

The observation of mumps-specific T-cell proliferation in two out of three low-titre donors suggests that these subjects were successfully primed by vaccination but seem to have lost circulating antibodies and memory B cells over the years. Our previous data on mumps-specific lymphoproliferation indicated that memory T cells, as revealed by lymphoproliferation assays, persist over a longer period of time following vaccination compared with circulating antibodies.[16]

The current concept of long-term protection is that long-lived memory B cells and long-lived plasma cells are two independent entities, with long-lived plasma cells able to survive for several decades independently of the presence of memory B cells of the same specificity.[20] In mumps, longevity of protection following vaccination seems limited compared with other vaccines such as vaccinia. The absence of both circulating antibodies and circulating memory B cells may indicate that individuals become susceptible again to infection with mumps virus and clinical disease. When antibody levels decline progressively or even disappear completely because of a lack of recurrent exposure to wild-type virus or revaccination, protection will depend on the speed with which the immune system is able to mount a protective antibody response that will prevent the virus from invading the human host. In the case of mumps, it is possible that the balance between mumps virus replication and the speed at which our immune system can produce high-quality

antibodies in response to encounters with the wild-type mumps virus is in favour of the wild-type mumps virus. We noted in the chimeric mouse model that the appearance of human IgG against mumps virus was slower than the anti-measles IgG response (data not shown). This difference in the kinetics of the antibody responses in the human PBL-SCID model may be attributable to the lower numbers of circulating mumps-specific B lymphocytes as compared with measles-specific memory B cells. However, in one of the early clinical trials with the Jeryl Lynn vaccine, antibody titres measured at 4 weeks following the administration of the vaccine were lower than those measured 5 weeks after mumps immunization.[21] Other studies have also noted the slow development of neutralizing antibodies after immunization.[22] The slow reaction of the immune system could be an indication that the current mumps vaccine strains are not as immunogenic as previously thought. This could result in a slower development of protective antibody titres and a weaker signal for the induction of memory B cells. Individuals without circulating antibodies may therefore be at increased risk of infection and clinical disease upon encounter with the wild-type virus. The findings of this study, along with the observation of waning mumps immunity in twice-vaccinated individuals, could explain why an outbreak of mumps occurred in twice-immunized individuals in the USA in 2006.[2]

Some investigators argue that waning immunity does not imply a loss of protection because of the presence of long-lived mumps antigen-specific cellular immune responses.[23,24] However, it is noteworthy that in this study the lymphoproliferation of T cells was similar in the two groups, irrespective of the presence or absence of circulating antibodies and memory B cells. This observation adds to the evidence showing that memory T cells do not play a major role in protection against infection when antibody levels have dropped below a critical protective level. It is most likely that mumps-specific antibodies play the leading role in protection against infection and disease.[16]

A major, albeit obvious, limitation of the present study is the fact that only PBMC were analysed and transferred into NOD-SCID mice. Our conclusions therefore only pertain to 'circulating' virus-specific memory B cells. We cannot exclude the possibility that mumps-specific memory B cells reside in the bone marrow or in secondary lymphoid organs and may be mobilized from these sites to contribute to protection following exposure to the wild-type virus. Mamani-Matsuda *et al.* showed, in a study in vaccinia-vaccinated individuals, that the spleen is the major niche for long-lived memory B cells and that the ratio between the number of memory B cells in the circulation and the number residing in the spleen is about 1 : 200.[20] To test for memory B cells residing in secondary lymphatic organs, subjects without circulating antibodies and without memory B cells could be challenged by giving them a mumps-containing vaccine, and the avidity of the induced antibodies could be tested shortly after vaccination (e.g. 7 days) and after a longer period of time (6–12 weeks). A rapid induction of antibodies with high avidity could indicate the presence of memory B cells outside the circulation.

In conclusion, in vaccinees without detectable serum antibodies against mumps virus, no or very limited numbers of memory B cells against these viruses seem to be present in the circulation. The absence of circulating antibodies as well as memory B cells may render these individuals susceptible to infection and clinical disease upon encounter with the wild-type mumps virus.

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Disclosures

None of the authors have a potential conflict of interest to declare regarding this paper.

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