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IgG Mediated Suppression of Immunological Memory

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SAMMANFATTNING

Antikroppar har inte bara förmågan att känna igen och neutralisera hot mot kroppen, utan kan också reglera immunresponsen mot sitt specifika antigen. Det är väl känt att Immunoglobulin (Ig) G administrerat tillsammans med röda blodkroppar inducerar >99% suppression. Detta har använts för att förhindra hemolytisk sjukdom hos nyfödda sedan 60-talet, men man har fortfarande inte helt förstått mekanismen bakom fenomenet. IgG-effekten på primärresponsen är väl etablerad, men huruvida det också påverkar det immunologiska minnet är fortfarande omdebatterat. I den här studien har splenocyter från immuniserade möss överförts till individer vars egna immunförsvar slagits ut med hjälp av gammastrålning. Detta genomfördes för att bevaka effekten IgG har på det immunologiska minnet utan störningar från primärresponsen. Parallellt med detta utfördes även två *in situ* experiment med olika immuniserings-doser. Antikroppsnivåerna i insamlat blodserum bestämdes med ELISA. När mössen blev immuniserade med $5x10^6$ röda fårblodkroppar observerades en 10-faldig suppression av minnesresponsen i det adoptiva cell överföringsexperimentet. Möss i *in situ* experimentet som immuniserades med den lägre dosen visade full suppression av immunologiska minnet medan den grupp möss som immuniserades med den högre dosen visade en minnesrespons lika hög som den positiva kontrollgruppen som immuniserats endast med de röda fårblodkropparna. Studerar man deras respektive primärrespons ser man att för att en komplett suppression av det immunologiska minnet ska kunna ske måste även primärresponsen visa total suppression. Detta leder oss till slutsatsen att induceringen av minnesresponsen beror på primärresponsen.

ABSTRACT

Apart from recognizing and neutralizing threats, antibodies also have the ability to regulate immune responses against their specific antigen. IgG administered together with erythrocytes is well known to induce >99% suppression. This has been used to prevent haemolytic disease in new-borns since the 1960's, but the mechanism behind it is still not completely understood. The effect on the primary response is well established, but its influence on induction of immunological memory is still heavily debated. In this study adoptive cell transfer of splenocytes to irradiated mice was used to survey the effect IgG has on induction of immunological memory without disturbance from the primary IgG response. Alongside this, an *in situ* boost experiment with two different priming doses was performed and antibody levels in serum detected using ELISA. When mice were primed with 10µg IgG-anti-SRBC (Sheep red blood cells) + $5x10^6$ SRBC, a 10-fold IgG suppression of memory was observed when using adoptive transfer and complete suppression of induction of immunological memory was seen in *in situ* boosted mice when primed with a 10 times higher antigen dose. A comparison to the primary IgG response in all experiments showed that, to allow for complete suppression of memory induction, there must be a complete suppression of the primary response as well. This brings us to the conclusion that the induction of the memory response depends on the primary response.

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ABBREVIATIONS

BCR	B cell receptor
ELISA	Enzyme-linked immunosorbent assay
FcγRIIB	Fc gamma receptor II B
IgG	Immunoglobulin G
KLH	Keyhole limpet hemocyanine
OVA	Ovalbumin
PBS	Phosphate buffered saline
RhD	Rhesus D antigen
SRBC	Sheep red blood cells

INTRODUCTION

The adaptive immune system consists of two response types, which involve different parts of the immune system and have different functions when eliminating intruders; the cell-mediated immunity and the humoral immunity. *Cell-mediated immunity* defends the body from microbial infections that are undetected by circulating antibodies due to their proliferation inside host cells such as phagocytes. The destruction of infected cells or the microbe inside the phagocyte is mediated by T cells in order to stop the infection from spreading to neighbouring tissue. *Humoral immunity* is composed of antigen specific antibodies circulating in the body via blood and secreted mucous. Antibodies recognize, neutralize and destroy foreign substances and infections, as well as the microbes causing it, by the activation of different effector mechanisms.

Antibody feedback regulation

Apart from recognizing and eliminating threats, antibodies also have the ability to regulate the response against the antigen they bind to, both by endogenous production and passive administration. This is known as antibody feedback regulation and can be either completely suppressive or enhancing by up to a 1000-fold towards the antigen (Gustavsson et al. 2000). Immunoglobulin (Ig) M is known to specifically enhance the humoral immune response against the antigen it recognizes (Möller & Wigzell 1965; Henry & Jerne 1968; Wason 1973) and IgG can either enhance or suppress the specific response, depending on which antigen it is specific against. When administered together with its antigen, IgG specific for soluble proteins such as keyhole limpet hemocyanine (KLH) and ovalbumin (OVA) enhance the response (Coulie and Van Snick 1985; Heyman 2014) while IgG specific for particulate antigens such as erythrocytes effectively suppresses the specific antibody response (Möller & Wigzell 1965; Heyman & H Wigzell 1984; Karlsson et al. 1999).

IgG mediated suppression

The suppressive response mediated by IgG does not only act when administered in a close time relation to its antigen; an ongoing antibody response can actually be suppressed by IgG given up to 5 days after SRBC (Chan & Sinclair 1973; Henry & Jerne 1968; M. C. I. Karlsson et al. 2001). Suppression has been observed with all four subclasses of IgG in mice; IgG1, IgG2a, IgG2b and IgG3 (Heyman & Wigzell 1984). The ability of IgG to suppress immune responses against erythrocytes has been used clinically to prevent haemolytic disease in new-born since the 1960's (Clarke et al. 1963). If a rhesus D (RhD) negative woman carrying an RhD positive fetus is immunized with fetal erythrocytes through trans placental haemorrhaging or during delivery, her immune system will start producing antibodies against RhD, leading to the degradation of the fetus red blood cells during the next pregnancy. Human IgG anti-RhD antibodies administered passively to the mother usually within 72 hours of birth will suppress this response and inhibit haemolytic disease during the next pregnancy. Even though this method of medical aid has been used for decades, it is still not clear how this suppressive mechanism works.

Mechanism behind IgG mediated suppression

Currently there are three leading hypotheses to explain the suppressive mechanism, the first being co-cross linking of inhibitory FcγRIIB and B cell receptor (BCR) by IgG immune complexes leading to inhibition of B cell activation. This has been shown to take place *in vitro* (Amigorena et al. 1992; Muta et al. 1994) and is speculated to take place *in vivo* in a similar manner, where the antigen-specific B cells are negatively regulated by the cross linking of these receptors by IgG-antigen complexes. However, both $F(ab')_2$ fragments (Tao & Uhr 1966; Karlsson et al. 1999) and IgE anti-SRBC (Karlsson et al. 1999), neither of which can bind to any FcγR, have shown effective suppression. In other experiments where FcγRIIB deficient mice were administered with IgG the response against erythrocytes were suppressed as effectively as in wild-type mice, suggesting that suppression does not depend on FcγRIIB (Karlsson et al. 1999; 2001). The second explanation is that IgG-antigen complexes are disposed by phagocytosis and therefore unable to interact with the immune system. However, since FcγR deficient mice also showed suppressed response when given antigen specific IgG, any elimination must be independent from these receptors (Karlsson et al. 1999). The third and currently most likely hypothesis is the one of epitope masking. The activation of B cells would simply be prevented due to the antigen being disguised from B cell recognition when bound to IgG antibodies. This fits very well with the majority of experimental data such as the independence of FcγRs and that IgE and $F(ab')_2$ have the ability to suppress. This is however very hard to show experimentally and waits to be directly proven.

The effect on the primary antibody response when IgG is passively administered is well documented (Heyman 2014), but there are different results have been reported when it comes to whether it also affects the induction of immunological memory. Some studies show that when primed with IgG and antigen together, the induction of memory is suppressed in the same manner as the primary immune response (Nicholas & Sinclair 1969; Dresser 1990) or suppressed but not to the same extent as the primary response (Heyman & Wigzell 1985) while others show that it has no effect on memory responses at all (Safford & Tokuda 1971; Brinc et al. 2008).

Scope of this investigation

Because of these incoherent reports we decided to perform our own experiment on the subject in order to determine the level of memory suppression. One way to test the impact on immunological memory, while eliminating the risk of endogenous suppression by IgG anti-SRBC produced during the primary response, is by adoptive cell transfer. With this process the recipient group acquires the immunity against the antigen the donating group has received without ever having been exposed to it.

We performed an adoptive cell transfer experiment alongside two *in situ* boost experiments with different boost doses, with the aim to investigate impact on immunological memory in an undisturbed environment.

MATERIALS AND METHODS

Mice

BALB/c mice were purchased from Bommice (Ry, Denmark) and kept at the National Veterinary Institute, Uppsala, in animal facilities. The Uppsala Animal Research Ethics Committee approved all animal experiments performed to gather material for this study.

Antibodies

Polyclonal IgGb anti-SRBC was prepared from serum collected from CB17 or C57BL/6 mice and purified using Protein-A sepharose columns (Amersham Pharmacia Biotech, Uppsala, Sweden) followed by dialysis against PBS, sterile filtration and storage at -20°C until use.

Antigens

SRBC (Håtunalab AB, Håtunaholm, Sweden) stored in Alsever's solution at 4°C were washed in PBS three times before use.

Primary immunization and blood sampling

Polyclonal IgG anti-SRBC and SRBC in 200 µl PBS was injected into the mice lateral tail veins. SRBC was injected 30 minutes past the IgG anti-SRBC immunization. Positive controls received SRBC alone and naïve mice were used as negative controls. The ventral tail artery was used to collect blood samples. Immunization doses are specified in the figure legends.

Boost immunization after adoptive cell transfer or *in situ*

Irradiation treatment (7 Gy) was performed on the recipient naïve mice which were then rested for 24 h before adoptive cell transfer and antigen boost. Six months after the antibody and antigen priming the spleens from all three groups of mice were collected. Single cell suspensions were prepared by smashing the spleens through a nylon cloth. The cell suspension were washed in Hank´s balanced salt solution (HBSS) and fifteen million nucleated spleen cells in 200 µl HBSS were injected in one of the lateral tail veins prior to an injection of $5x10^5$ SRBC in 200 µl PBS. In the in situ experiments the mice received boost immunizations in the same manner, but no adoptive transfer were performed.

ELISA

Coating of plates

Fifty µl of 0.25 µg/ml poly-L-lysine (P1274 Sigma, St. Louis, Missouri, United States) in distilled H_2O were added to microtiter plates (Costar) and incubated for 45 minutes in 37°C. Plates were then washed 3 times in PBS, 100 µl of 0.25% SRBC in PBS was added, and plates were incubated at room temperature for 1 hour. Gentle submersion of the plates into 0.25% glutaraldehyde (Sigma) in PBS for 10 minutes was used to fix the erythrocytes and loose cells were removed by washing in PBS two times. Plates were blocked by the addition of 150 µl of a PBS solution containing 50 mg/ml dry milk and 0.02% NaN_3 and plates kept at 4°C until used.

Assay

Blocked plates were washed two times in PBS and 50 µl of sample diluted in dilution buffer (PBS + 0.05% Tween-20; WVR, Pennsylvania, United States; 0.25% dry milk and 0.02% NaN_3) to 1:25 and stepwise times two until 1:819,200 (SRBC) and 1:5 in the same manner until 1:163,840 (IgG + SRBC and naïve). Plates were then incubated over night at 4°C and washed 3 times in PBS + 0.05% Tween. Fifty µl of sheep anti-mouse IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, Sacramento, California, United States) diluted 1:1000 in dilution buffer were added and incubated in room temperature in humid chamber for 3 hours. This was followed by washing 3 times in PBS + 0.05% Tween and 2 times in PBS, and the addition of 100 µl of a substrate solution containing 1 phosphatase substrate tablet (Sigma) per 5 ml substrate buffer (44.4 M dH_2O, 1.012 M diethanolamine, 0.001 M $MgCl_2x6\ H_2O$, titrated with 5 M HCl to pH 9.8). After 30 minutes of incubation in the dark, OD was measured at 405 nm and the data analyzed with SoftMax software (Molecular Devices, Toronto, Canada).

The baseline was established by taking the average of the normal serum OD_{405nm} plus two times the standard deviation. Titers were determined as the dilution prior to the first dilution with an OD_{405nm} below the baseline.

Statistical analysis

A two-tailed, non-parametric Mann-Whitney t-test was used in order to determine the statistical difference between groups, with the statistical significance levels set as following: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. In cases where only two measuring points were available, a third with a value of 1 was added so the test could be performed.

RESULTS

In situ memory response

Two *in situ* experiment were performed on BALB/c mice, where one set of mice were primed with 10 µg IgG + $5x10^6$ SRBC or $5x10^6$ SRBC alone and one set of mice primed with 50 µg IgG + $5x10^7$ SRBC or $5x10^7$ SRBC alone 6 months prior to a $5x10^5$ SRBC boost. ELISA screening for IgG anti-SRBC detected present levels in blood serum, collected during three separate time points between day 21-49 after boost. When receiving the lower priming dosage, only the SRBC group showed induction of memory response (figure 1A). In the mice receiving IgG + SRBC, the levels of serum IgG anti-SRBC were as low as the group of mice receiving booster alone indicating an almost complete suppression. However, with a higher priming dose, the IgG + SRBC group showed no sign of suppression, with an IgG anti-SRBC level equally high as the SRBC alone group (figure 1B).

Adoptive transfer memory response

In parallel to this, an adoptive transfer method was used to avoid suppression of recall responses caused by IgG anti-SRBC from the primary response. Donor mice were primed with 10 µg IgG + $5x10^6$ SRBC or $5x10^6$ SRBC alone 6 months prior to spleen harvest and adoptive transfer. A single cell suspension containing $15x10^6$ nucleated splenocytes was transferred into irradiated recipient mice, in combination with a boost of $5x10^5$ SRBC. Blood serum was collected during 6 time points from 7 days prior boost until 121 days post boost, and IgG anti-SRBC levels were determined by ELISA. The adoptive transfer mice showed memory response in both the IgG + SRBC and SRBC groups (figure 1C). However, the response in IgG + SRBC was suppressed by a tenfold compared to the SRBC group.

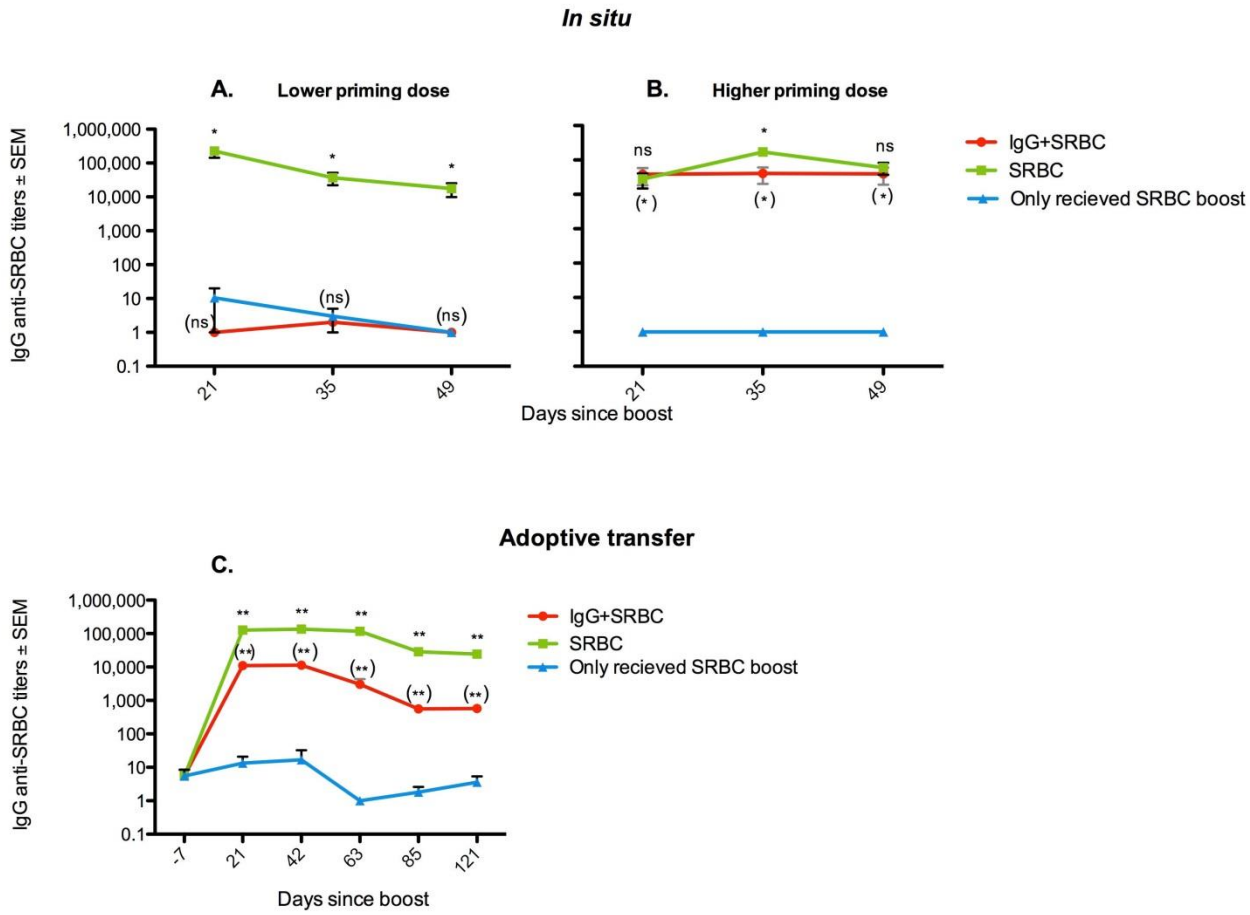

Figure 1. IgG impact on immunological memory. A) BALB/c mice primed with 10 µg IgG anti-SRBC + $5x10^6$ SRBC or $5x10^6$ SRBC alone, and a naïve group were boosted with $5x10^5$ SRBC 6 months after priming. B) BALB/c mice immunized with 50 µg IgG anti-SRBC + $5x10^7$ SRBC or $5x10^7$ SRBC 6 months prior to boost of $5x10^5$ SRBC. C) BALB/c mice were primed with 10 µg IgG anti-SRBC + $5x10^6$ SRBC or $5x10^6$ SRBC alone 6 months prior to spleen removal. A negative control group of naïve mice were used as well. A single cell suspension (containing $15x10^6$ nucleated splenocytes) made from the removed spleens were then injected intravenously together with a $5x10^5$ SRBC boost into naïve mice 24 hours after irradiation treatment (7 Gy). IgG anti-SRBC levels were determined in all experiments by ELISA using blood serum collected at indicated time points. SRBC immunized group was analysed with a serum dilution of 1:25 and then stepwise by two until 1:819,200, IgG + SRBC and naïve groups with a dilution of 1:5 to 1:163,840. Titers were determined as the dilution prior to the first dilution with an OD405nm below the baseline and are presented as log10 on the y-axis. In experiment A, n = 5-7/group and in experiment B, n = 2-6/group. A Mann-Whitney U-test was used to detect statistical differences between IgG + SRBC and SRBC groups (without parentheses) and IgG + SRBC and naïve groups (with parentheses): ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

DISCUSSION

Several studies have been made on the impact of IgG on immunological memory, but the effect of endogenous IgG generated from the primary response had never been excluded. In this study we wanted to record the level of suppression of memory in an undisturbed environment and therefore adoptive transfer was used.

When measuring the IgG response to antigen in blood it can be problematic to distinguish the antibodies administered from the ones produced *in vivo*. One way to work around this is by administering antibodies of another allotype than the one produced by the animals own immune response. Using an ELISA method that discriminates between allotypes allows an accurate detection of the antibody response to the immunization rather than to the antibodies administered. In this case however, we waited for 6 months before boost, in order for the endogenous IgG anti-SRBC levels in the bloodstream to go down.

Interestingly, the *in situ* experiment where the mice received the higher priming dose showed no suppression on memory in contrast to the complete suppression seen in connection to the lower dose. If we look at the primary IgG anti-SRBC response (Bergström et al. Unpublished data) in these two experiments, we see that the mice receiving the lower priming dose have a primary response that is completely suppressed, whereas the ones receiving the higher priming dose show a response around day 21. In the IgG + SRBC group in the higher priming dose experiment there were two extreme outliers; one with a really high response and one barely responding at all. This obviously affects the main outcome, and the experiment would have to be repeated to establish more reliable results.

In the adoptive transfer experiment, the IgG + SRBC group showed a suppressed memory response by around a 10-fold compared to the levels of the SRBC group. The primary IgG response in this experiment showed an initial suppression which turned into an increasing response around day 21. Something seen in all of these experiments is that in order to allow for complete suppression of the induction of memory response, the primary response must be completely suppressed as well. This brings us to the conclusion that the memory response depends on the primary response.

So how come the primary response differs between the experimental groups? The difference on suppression in the *in situ* groups could be due to the differing priming doses administered. Levels of suppression have been shown to correlate with the amount of administered IgG, where a higher dose equals a higher suppressive response. (Henry & Jerne 1968; Heyman & Wigzell 1984) However, in these previous experiments varying doses of IgG were administered together with a constant dose of SRBC. Our experiment provides data on the suppression after the administration of an increased dose of both SRBC and IgG which could bring new insights into the suppressive impact depending on proportions between antibody and antigen. In our higher dose experiment, only a 5 times higher dose of IgG anti-SRBC was administered together with a 10 times higher dose of SRBC, suggesting that the amount of administered antibody was not enough to effectively induce a complete suppression.

One explanation for the incoherence in the previous results regarding IgG impact on immunological memory may be that most studies only measured the primary IgM response, assuming that a complete suppression of primary IgM meant the same on primary IgG. However IgM responses are much more easily suppressed than IgG responses, as shown in these experiments and by Bergström et al. (unpublished data) where a complete suppression of primary IgM responses not necessarily meant a complete suppression of the IgG response. In the studies where primary IgG were detected, samples were taken within a close time frame after the immunization. However, in this study we see an IgG response around three weeks after immunization, so samples collected before this might show a complete suppression even though it may only be initial.

In conclusion, this study showed that the extent of memory suppression is dependent on the primary response, although further investigations should be performed in order to confirm these findings. A greater understanding of how memory suppression operates may lead to advantages within clinical IgG treatment to prevent haemolytic disease in new-born. Currently the polyclonal antibodies specific against RhD are prepared from large amounts of human sera. Given enough insight into the mechanism, monoclonal IgG anti-RhD antibodies could be produced and the risks of infection decreased.

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