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# Evidence in favor of a role of idiotypic network in autoimmune hemolytic anemia induction: theoretical and experimental studies

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*Keywords*: autoimmune anemia, immune network, mathematic model, humoral kinetics

## Abstract

**Formation dynamics of antibodies to rat erythrocytes (REs) and auto-antibodies to mouse erythrocytes were studied in an experimental model of autoimmune hemolytic anemia (AHA) in mice. The experimental conditions of AHA were simulated in a mathematical model of an immune network. It was found that maximal production of auto-antibodies and antibodies to REs do not synchronize. Antiserum, obtained at the peak of auto-antibodies formation, competed with REs for bounding with antibodies. This represents proof that auto-antibodies to erythrocytes and antibodies to REs are an idiotype–anti-idiotypic pair. In the autoimmune reaction, the autoreactive clone, being anti-idiotypic, responded earlier than the clone reacting to the injected antigen. Comparison of autoimmune reaction kinetics in the mathematical model of an immune network with experimental dynamics of AHA shows them to be similar. So activation of the autoreactive clone to erythrocytes during experimental AHA in mice is mediated by idiotype–anti-idiotypic interactions with the clone reacting to REs.**

## Introduction

The experimental model of autoimmune hemolytic anemia (AHA) in mice, provoked by injection of rat erythrocytes (REs), is generally recognized. But it is still not clear how this autoimmune reaction develops. The existing explanations are based on the idea of molecular mimicry (1, 2). However, research carried out by Scott *et al.* (3) showed that mAbs to mouse erythrocytes (MEs), obtained from mice with spontaneous hemolytic anemia, did not cross-react with REs. Autoimmune response to erythrocytes of young (pre-autoimmune) NZB mice is controlled by anti-idiotypic CD 8+ suppressors (4). Autoimmune reaction may occur through activation of autoreactive lymphocytes by a foreign antigen via idiotype–anti-idiotypic interactions (5). Existence of idiotype–anti-idiotypic links between auto-antibodies and antibodies to foreign antigen has been demonstrated, although such pairs are few in number. For example, human mAbs to alpha-1,3-dextran—antigen of opportunistic *Enterobacter cloacae* and *Serratia liquefaciens*, bind to mouse mAbs to acetylcholine receptor of human muscles (6). The rheumatoid factor is bound as an idiotype–anti-idiotype with antibodies to streptococci antigens (7), antibodies to *Mycobacterium tuberculosis* (5) and antibodies to collagen (8). The similarity between the rheumatoid factor and proteins of herpes viruses, which bind an Fc gamma fragment, suggests that formation of the rheumatoid factor is an anti-idiotypic response to antibodies to these proteins and shows involvement of cytomegalovirus in pathogenesis of rheumatoid arthritis (9).

### *Hypothesis*

The mechanism mediated by idiotype–anti-idiotypic interactions involves kinetics of autoimmune reaction, which differs from those involved in the antigenic mimicry. When an autoimmune reaction is developed via idiotype–anti-idiotypic interactions in an experimental model of AHA, maximal production of antibodies to REs and auto-antibodies to MEs should not coincide, and the relation between the kinetic curves should look like dynamics of idiotypic and anti-idiotypic antibodies formation. In the case of immune cross over, the generation peaks for antibodies to inductor and antibodies to auto-antigen synchronize.

Discovery of the role of idiotype–anti-idiotypic interactions in development of autoimmune reactions can only be obtained through understanding of the mechanisms at work in immune network functioning. There are currently no clear ideas about network functioning and its role in regulation mechanisms. Study of the idiotype–anti-idiotypic network is a complex task, which is best addressed by use of mathematical modeling (10–12). We have already introduced

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a mathematical model of an immune network (13), which can be used to gain understanding of autoimmune reactions mediated via idiotype–anti-idiotypic interactions. So the aim of the work, which we are now reporting, was to study the kinetics of antibody response to REs and auto-antibodies to MEs in an experimental model of AHA in mice, modeling the experimental conditions on the mathematical model of an immune network.

## Methods

*Animals and induction of AHA*

Three-month-old male MRL mice were used. They were obtained from the Pushino breeding facility in Russia and housed under standard laboratory conditions with food and water at a constant temperature of 20 ± 5°C. The animals were given three intraperitoneal injections of intact REs ($2.8 \times 10^8$ cells in 0.5 ml): on days 0, 2 and 4. The day of anemia induction was designated as day 0. The animal experiments were performed in accordance with provisions of the local animal care commission.

*Samples*

Blood was obtained before immunization and on days 5, 10, 15, 20 and 25 following the primary dose. Ten mice were sacrificed every 5 days. Blood was collected and EDTA plasma samples were analyzed.

*Evaluation of AHA*

The development of hemolytic anemia was assessed every 5 days by measuring the red blood cells.

*Quantification of antibodies to REs*

Antibodies to REs were quantified by hemagglutination using intact REs. Mouse serum was warmed for 30 min at 56°C and titrated. Plate sockets were filled with 50 µl of serum and the same volume of 0.5% RE suspension and incubated at room temperature. Reaction results were registered in 24 h.

*Quantification of antibodies to MEs*

Incubation of intact MEs with the investigated serum did not give an agglutination reaction. Therefore, free auto-antibodies to MEs were quantified by indirect Coombs test. The test mouse plasma (dilutions ranging from 1/10 to 1/20 000) was warmed for 30 min at 56°C and then incubated for 2 h at 37°C with intact MEs (2% suspension). Following washing, monospecific anti-mouse IgG rabbit serum—provided by the firm ZAO Biosan (Novosibirsk, Russia)—was added and incubated at 4°C for 24 h. As a control, intact MEs were incubated with physiological solution, instead of tested serum, and later on with serum against IgG mouse. No agglutination was detected.

*Determination of idiotype–anti-idiotypic interactions between auto-antibodies and antibodies to heterologous erythrocytes in a hemagglutination inhibition reaction*

Mouse plasma from five animals obtained on the 15th day after immunization was pooled. Eighty microliters of pooled mouse plasma from the 15th day (dilutions ranging from 1/2 to 1/1024) was incubated with intact REs (0.5% suspension) and with 80 µl of pooled mouse plasma (from five animals) from the 10th day. As a control, the serum received on the 10th day was substituted by physiological solution or intact mouse serum. Results were registered in 24 h. Three experiments were carried out.

*Statistical analysis of the data*

Results were expressed as mean ± SD.

## Results

*Time course of antibodies to REs and MEs in mouse AHA*

Mice responded to RE injection by developing transitory autoimmune reaction to their own erythrocytes, which resulted in a reduced number of erythrocytes and increased number of auto-antibodies to ME. The formation dynamics of RE antibodies and ME auto-antibodies and changes in quantity of blood erythrocytes in immunized animals during 25 days after immunization are shown in Fig. 1.

Anemia development coincided with formation of auto-antibodies to erythrocytes, but not with generation of antibodies to REs. For example, minimal content of erythrocytes in mouse blood, as well as maximal production of auto-antibodies to erythrocytes were recorded on the 10th day after immunization. Erythrocyte levels in mouse blood had almost been restored by the time antibodies to REs reached their maximal level (Fig. 1).

It was shown that maximal generation of anti-rat and anti-mouse antibodies do not synchronize (Fig. 1). Peak production of auto-antibodies to erythrocytes was observed on the 10th day, while the maximum amount of antibodies to RE is generated on the 15th day after immunization.

Surprisingly, we found that production of auto-antibodies to erythrocytes and anemia development occurred more quickly than generation of antibodies to heterologous erythrocytes. This is in contrast to the expected result that auto-antibodies would be generated after antibodies to RE.

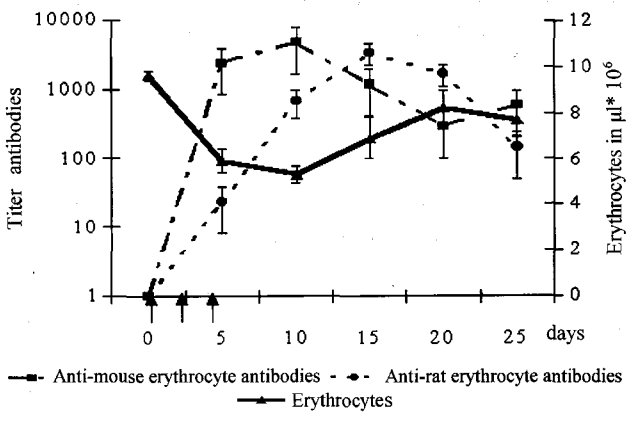


**Fig. 1.** Formation kinetics of auto-antibodies to ME, antibodies to RE and erythrocyte quantity in blood of mouse with experimental AHA. Injection of REs (arrow). Each data point represents the mean of 10 mice.

*Idiotypic nature of interaction between RE antibodies and auto-antibodies to erythrocytes. Inhibition of agglutination of REs by serum containing antibodies to MEs*

Antiserum obtained at the peak of auto-antibodies production (10th day) was tested in a hemagglutination inhibition reaction in a test system consisting of RE and antiserum, which was obtained at the time of maximal generation of antibodies to REs (on the 15th day). Fig. 2 shows results of three experiments, in each of which the test serums were produced by pooling of serums from five animals.

Antiserum, obtained on the 10th day, inhibited agglutination of REs to a considerable extent (Fig. 2). For example, in the first experiment, the titer of RE antibodies was 1/128 in control, where immune serum was substituted by physiological solution, and was 1/64 in control, where immune serum was substituted by intact mouse serum; the titer of RE antibodies was 1/4 with present ME auto-antibodies. It follows that antiserum containing auto-antibodies competes with RE for binding to their antibodies, i.e. auto-antibodies bind to idiotypes of RE antibodies. This proves that RE antibodies and ME auto-antibodies make an idiotype–anti-idiotypic pair.

*Mathematical model of an immune network: computer modeling of AHA*

Autoimmune reaction was studied theoretically in a mathematical model of an immune network, based on idiotype–anti-idiotypic interactions. The model was constructed on the basis of Jerne's theory of anti-idiotypic interactions in the immune system and the well-known phenomenology of immune reactions (14, 15). This model is a system of discrete equations, describing an idiotypic network with a certain geometry. The basic element in the model is a lymphocyte clone with an idiotype (Id) and a number, which distinguishes it from other clones (Id1, Id2, ..., Id*n*). According to Jerne's theory, clones interact anti-idiotypically, i.e. IgRs, free antibodies or TCRs of one idiotype bind to the corresponding IgR, antibodies or TCR of another idiotype, as with an antigen (14). In our model, all clones participate in such interactions forming a continuous network. Its geometry can be defined randomly. In the simplest case, it is a linear chain, where every idiotype has two clones with anti-idiotype. The length of the chain is optional, clones are numbered in order from the first to the last; the last clone is bound to the first, closing the chain and forming a ring.

In every time point, a lymphocyte clone has a certain 'activity' value (*U*). Lymphocyte activity in the model is (quantitatively expressed) the ability to react to an activation signal and transmit this signal to other cells via anti-idiotypic interactions. In a real system, this would correspond to higher proliferative and functional activity of the corresponding cells. For example, B-cells have the following activity functions: intensity of proliferative response, amount of produced antibodies and formation of memory cells during the immune response.

According to Jerne's theory, bonds between clones in the model must have the following properties:

(1) Interacting clones are antigens to each other and can stimulate each other. Such bonds are referred to in the model as 'direct' bonds (Fig. 3). Bond (*F*) depends on activity (*U*) of the idiotype (Id*i*) and anti-idiotype (AId*j*); *k*1 is a coefficient, determining the interaction effect (e.g. amount of antigens), and $\alpha$ is affinity of the interaction with the antigen.

(2) Clones can also have specific interaction via a system of indirect bonds (*F*2), organized with the help of cellular activity 'products'—antibodies or sensibilized lymphocytes (Fig. 3).

According to Jerne's theory, an indirect bond is negative, i.e. it limits idiotype activity. But experimental data show that effects of interaction can be both positive and negative. Our view is that the value and sign of the effect in a system of indirect bonds will depend on the cellular activity. A clone with idiotype reduces activity of a clone with anti-idiotype, if the second is highly active. On the contrary, if activity of a clone with anti-idiotype is low, it increases. So direct bonds (*F*1)

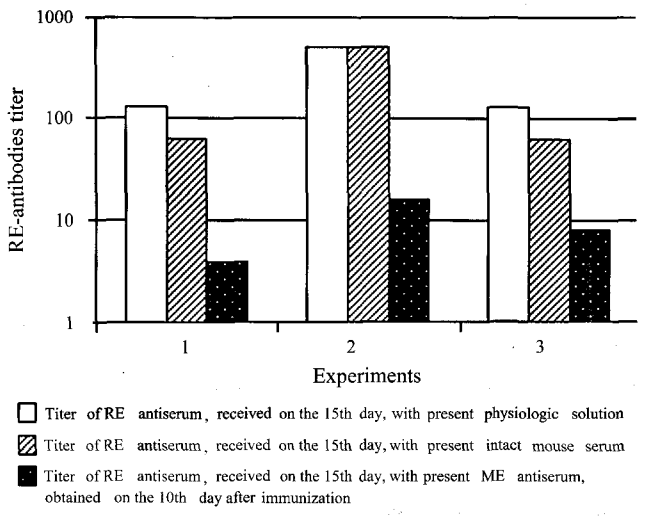


**Fig. 2.** Hemagglutination inhibition of REs with present auto-antibodies. Data obtained from three experiments. In each of them, the investigated serums were produced by serum pooling of five mice.

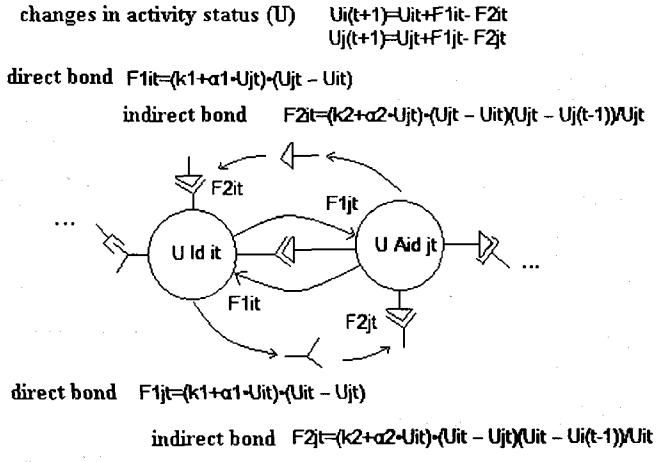


**Fig. 3.** The scheme shows idiotypic interactions between cells in the model and their mathematical description. One pair of cells interacting in a linear closed chain is represented.

are always activating (positive), while indirect bonds can be both positive and negative. The sign of binding depends on the cellular activity (Fig. 4).

The consequence of what we have said is that equations of interaction between clones *i* and *j* can be written as follows:

Direct bond: $F1it = (k1 + \alpha1 \bullet Ujt) \bullet (Ujt - Uit)$;
Direct bond: $F1jt = (k1 + \alpha1 \bullet Uit) \bullet (Uit - Ujt)$;
Indirect bond: $F2it = (k2 + \alpha2 \bullet Ujt) \bullet (Ujt - Uit)(Ujt - Uj(t - 1)/Ujt$;
Indirect bond: $F2jt = (k2 + \alpha1 \bullet Uit) \bullet (Uit - Ujt)(Uit - Ui(t - 1)/Uit$ and
Changes in activity status ($U$): $Ui(t + 1) = Uit + F1it - F2it$ and $Uj(t + 1) = Ujt + F1jt - F2jt$.

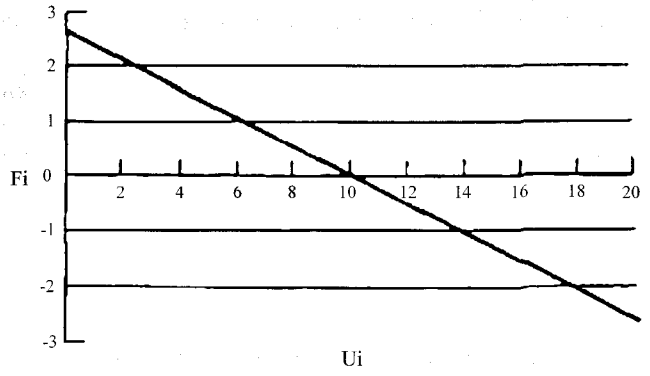


**Fig. 4.** Dependence of $Fit - (F1it - F2it)$ on $Uit$ at preset values $Ujt$ and $Uj(t - 1)$.

All these bonds are observed simultaneously. Network status is defined as the outcome of interactions between clones. For each activity status, there is a certain resulting effect of all bonds at a given moment. In the absence of a signal (antigen), a steady state is established as a result of interaction between clones for an unspecified period of time. The level of activity remains constant. This status can be referred to as 'initial (or virgin) network condition' (11).

As the model is non-linear, a large number of such conditions is supposed. Cells interact in time ($t$). As a conventional time unit, we take one stage of interaction, allowing transfer of an antigenic signal from an initially activated clone to one element of the network. In the next (second) time point, the signal will be transmitted to the next element of the network and the next interaction stage will develop in the initial pair. So, at one time point, all 'neighbors' of the network interact with each other and their activity changes in accordance with this interaction.

We studied experimental conditions of AHA in the framework of this model. Lymphocytes in the immune network are bound in idiotype–anti-idiotypic interactions. In a pair of such lymphocytes, one of the clones was marked as autoreactive, and the other one as a clone, which reacts to a foreign antigen. The initial state of the network was determined as a state of tolerance to a constantly present auto-antigen (Fig. 5). It was characterized by lower activity of clones, compared with their state before injection of the auto-antigen. Subsequent injection of an antigen interacting with a lymphocyte, which is anti-idiotypic with respect to an auto-clone, causes transitory autoimmune reaction (Fig. 5), which results in higher auto-clone activity. Clone activity conditions are shown in the diagram.

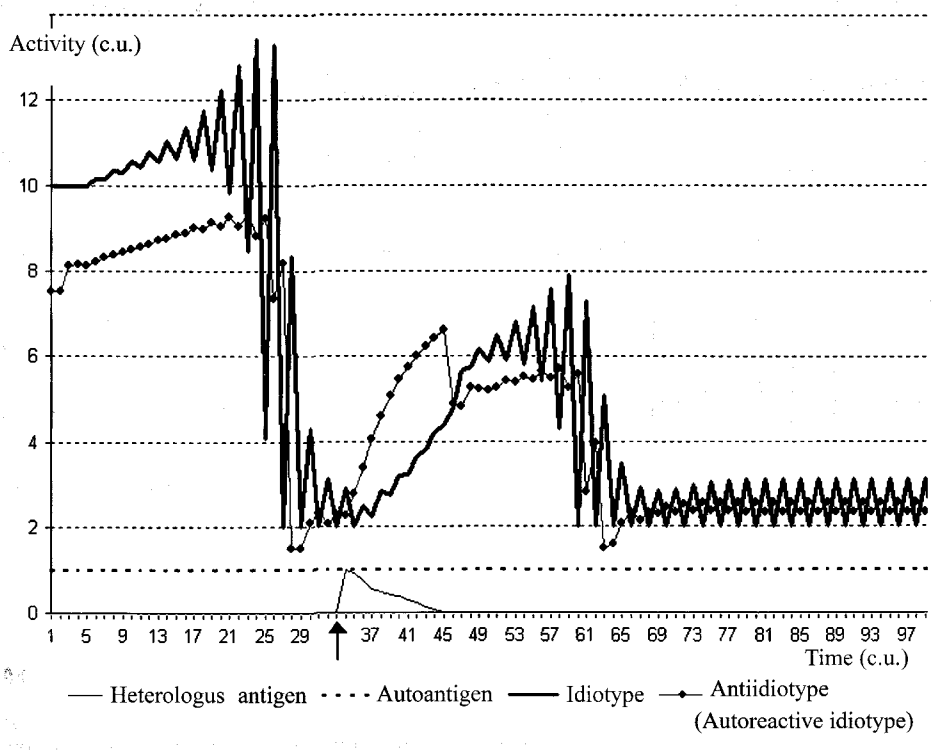


**Fig. 5.** Mathematic modeling of anti-idiotypic and idiotypic antibodies dynamics when heterologous antigen is injected (arrow).

It should be mentioned that an autoreactive clone, being anti-idiotypic, responds earlier than a clone reacting to an injected antigen. Comparing the obtained theoretical kinetics of autoimmune reaction (Fig. 5) with dynamics of AHA obtained during the experiment (Fig. 1), we see that they are similar.

## Discussion

The research on dynamics of antibody formation showed that maximal production of ME auto-antibodies and RE antibodies do not synchronize. The observed generation of auto-antibodies and antibodies to RE is similar to the dynamics of formation of idiotypic and anti-idiotypic antibodies during immune response, when peak production of anti-idiotypic antibodies usually coincides with minimal production of idiotypic antibodies (16). The observed kinetics of antibody formation is also similar to IgM and IgG kinetics during primary immune response when IgM and then IgG are initially formed. It might therefore be supposed that the observed kinetics was caused by production, initially, of IgM antibodies (on the 10th day) and, later, of IgG (on the 15th day), cross-reacting with MEs and REs. However, in the indirect Coombs test, we used the monospecific serum against IgG mouse, so the first peak of the antibody formation kinetics (on the 10th day) is represented by IgG. The antibodies to REs detected during the agglutination test can be IgG and IgM. But their maximum occurs later (on the 15th day) than the auto-antibodies maximum. Therefore, the kinetics of formation of antibodies to erythrocytes and antibodies to heterological erythrocytes in the AHA model could not be explained by the formation of cross-reacting antibodies belonging to different classes. Thus, (i) AHA in mice does not develop as a result of immune cross over, (ii) different lymphocyte clones react to mouse and REs and (iii) antibodies to RE and auto-antibodies to erythrocytes present an idiotype–anti-idiotypic pair.

Inhibition of RE agglutination in the presence of ME antibodies is a convincing proof that auto-antibodies to ME and antibodies to RE make an idiotype–anti-idiotypic pair (Fig. 2).

Compliance of experimental AHA dynamics with theoretical dynamics of autoimmune reaction, obtained in a mathematical model of idiotype–anti-idiotypic interactions, is a ground for considering induction and development of AHA to be mediated via idiotype–anti-idiotypic interactions.

The obtained data make it reasonable to suppose that lymphocytes to RE normally provide anti-idiotypic suppressor control over lymphocytes, which carry idiotypes against their own erythrocytes. This does not contradict Jerne's immune network theory, according to which anti-idiotypic lymphocytes suppress the function of the associated idiotype-positive lymphocytes (17) and support natural tolerance and low level of auto-antibodies (4, 12, 18). Activation of lymphocytes by REs, which are anti-idiotypic with respect to idiotype of autoreactive clone, provokes AHA. A situation when natural tolerance is disturbed by an antigen binding to lymphocytes, which are anti-idiotypic with respect to autoclones, has already been described in the mathematical model of Sulzer *et al.* (12).

The fact that auto-antibodies form earlier than antibodies to the injected antigen was unexpected. Early formation of auto-antibodies, anticipating formation of antibodies to the injected antigen, could be a sign of pre-existing asymmetry in idiotype–anti-idiotypic interactions between an autoreactive clone and a clone, reacting to foreign elements. The reason for such asymmetry is that autoreactive clones constantly experience an activation signal from the body's auto-antigen, unlike associated anti-idiotypic lymphocytes, which do not experience constant pressure from an external antigen. This asymmetry, as a condition, was simulated in the mathematical model by auto-antigen injection (Fig. 5). As a result, we obtained theoretical curves corresponding to the experimental curves. So activation of lymphocytes, which are anti-idiotypic with respect to the idiotype of an autoreactive clone, is what initiates and provokes development of AHA in mice after heterologous erythrocyte injection, and such development is not caused by mimicry between foreign and own antigens.

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## Abbreviations

| | |
|---|---|
| AHA | autoimmune hemolytic anemia |
| ME | mouse erythrocyte |
| RE | rat erythrocyte |

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