Long-Term Suppression of Natural and Graft-Induced Xenophile Antibodies by Short-Term Antigen-Cyclophosphamide Treatment

A. Breitkreuz, K. Ulrichs, V. Eckstein, and W. Müller-Ruchholtz

NATURAL xenophile antibodies (NXA) and graft-induced xenophile antibodies (GIXA) are considered major barriers to organ transplantation between phylogenetically discordant species.[1] Thus, major efforts to achieve successful xenotransplantation must aim at (a) the elimination of NXA from the recipient's serum and (b) efficient inhibition of subsequent GIXA production after xenogeneic sensitization. This study investigates the effects of a time-limited treatment consisting of xenogeneic antigen (AG) plus short-term cyclophosphamide (CY) in the model "human-to-rat" to eliminate preexisting NXA and to inhibit graft-dependent GIXA production. This approach is based on previous studies in our laboratory[2], in which B-lymphocyte tolerance was experimentally achieved in rats by applying CY at the most vulnerable phase of cell proliferation, that is, during xenogeneic sensitization. The principles of this concept are presently being successfully applied in the clinic by treating severe forms of autoimmune diseases, such as systemic lupus erythematosus.[3]

MATERIALS AND METHODS

Animals

Lewis rats (3-month-old females; MHC:RT1^{l}) received 5×10^6 human peripheral blood lymphocytes (PBL) as xenogeneic AG intraperitoneally either on day 0 or on day 0 plus day 30. Cy was applied at a dosage of 20 mg/kg for 6 days, (a) from day 0 until day 5 or (b) additionally from day 30 until day 35. The total dosage of 120 mg/kg represented approximately 65% LD_{50}.

Experimental Design

The experimental design consisted of four treatment groups: group 1 received CY from day 0 until day 5; group 2 received a single application of xenogeneic AG on day 0; group 3 received AG on day 0 and CY from day 0 until day 5, whereas group 4 was additionally treated with AG on day 30 and with CY from day 30 until day 35. Tail vein blood was collected at 10-day intervals for analysis of NXA and GIXA by standard flow cytometry (FACS).

Determination of NXA and GIXA in Indirect Immunofluorescence

Rat sera were diluted 1:2, 1:4, 1:8, etc for the determination of IgM and IgG antibody titers in indirect immunofluorescence (FACS) using human PBL as target cells (Fig 1). Secondary antibodies were (a) fluorescein (DTAF)-conjugated, AffinePure goat anti-rat IgG, Fc fragment specific (cat. no. 112-015-071, Dianova, Hamburg, Germany), and (b) fluorescein (DTAF)-conjugated AffinePure F(ab')2 fragment goat anti-rat IgM, Mu chain specific (cat. no. 112-016-075, Dianova). The titer was determined by the highest serum dilution at which 90% or more antibody binding was observed above background fluorescence.

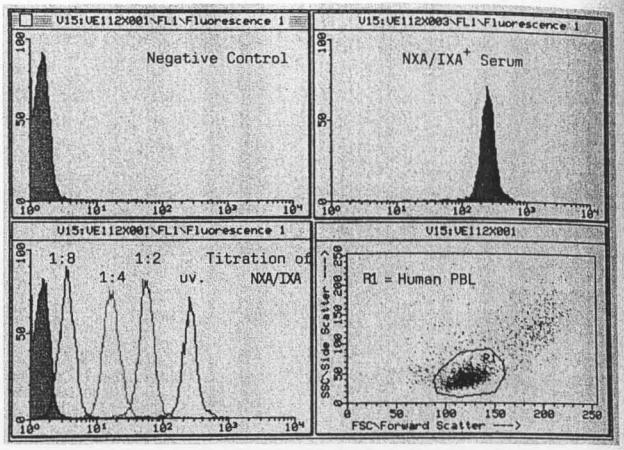

Fig 1. Analysis of NXA and GIXA (previously called IXA) by flow cytometry (FACS) and determination of antibody titers. **(Top left)** Negative control. **(Top right)** Example of an NXA- or a GIXA-positive rat serum. **(Bottom left)** Titration of an NXA- or a GIXA-positive rat serum. **(Bottom right)** R1 = human PBL, which served as target cells.

Specificity Control

To test for the specificity of suppressive effects after combined treatment, selective rat sera were comparatively tested on human, porcine, and fish (trout) target cells.

RESULTS

NXA Reduction by CY (Group 1)

To test whether it is possible to suppress the production of NXA in nongrafted animals, normal, that is, unsensitized Lewis rats received CY from day 0 to day 5 (n = 5). The result of this experiment is documented in Fig 2 and can be summarized as follows: the CY treatment significantly inhibits NXA production. However, the inhibition is reversible and lasts only as long as CY is effective in vivo.

GIXA Induction by Xenoantigen (Group 2)

The following experiment was designed to establish the relevant positive control in our model "human-to-rat" for a subsequent manipulation of NXA and GIXA production.

From the Institute of Immunology, Medical School of the University, Kiel, Germany.

Address reprint requests to Dr Karin Ulrichs, Institute of Immunology, Medical School of the University, Brunswikerstrasse 4, D-2300 Kiel, Germany.

0041-1345/93/$3.00/+0

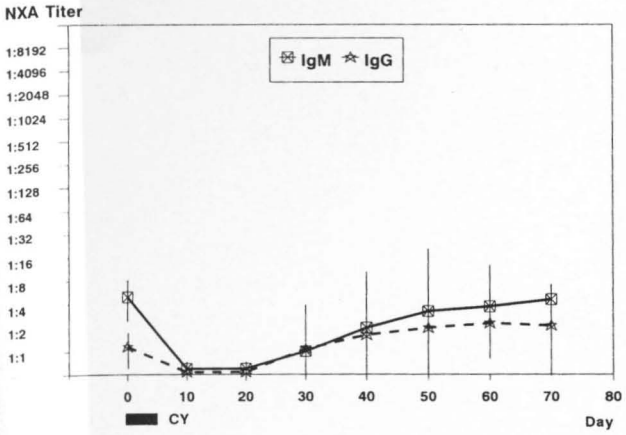

Fig 2. NXA reduction by CY (group 1). Normal, that is, unsensitized Lewis rats received CY from day 0 to day 5 (n = 5 per day).

Normal Lewis rats received a single injection of AG on day 0 (n = 5). The result is documented in Fig 3 and can be summarized as follows: the production of GIXA strongly increases and, as expected, switches from IgM to IgG isotype. This xenogeneic sensitization effect lasts for a long period of time.

NXA/GIXA Reduction by Xenoantigen and CY (Group 3)

To answer the question, whether a time-limited combined treatment, consisting of AG and CY is capable of suppressing not only GIXA, but also NXA, Lewis rats were treated with AG on day 0 and with CY from day 0 to day 5 (n = 5). The results of this experiment are documented in Fig 4 and can be summarized as follows. (a) Effects of the combined treatment of NXA (in comparison with group 1, see Fig 2): NXA-IgM are significantly reduced, whereas the combined treatment has no significant effect on NXA-IgG. (b)

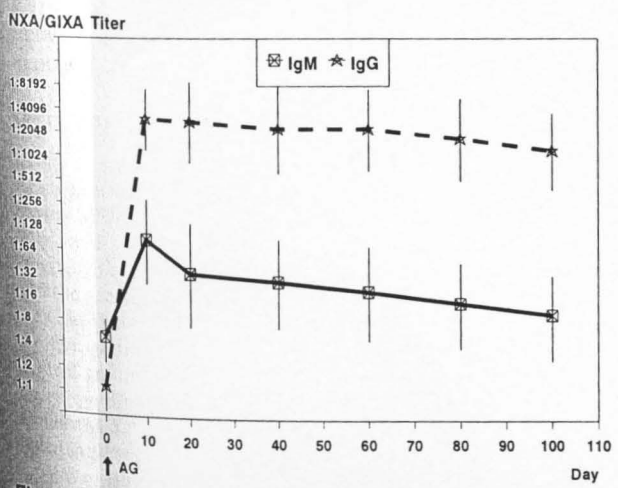

Fig 3. GIXA induction by xenoantigen (group 2). Normal Lewis rats received a single injection of xenoantigen (AG) on day 0 (n = 5 per day).

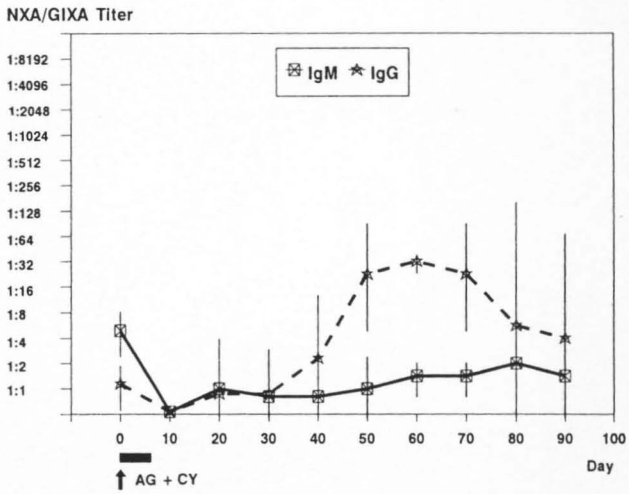

Fig 4. NXA/GIXA reduction by xenoantigen and CY (group 3). Lewis rats were treated with AG on day 0 and with CY from day 0 to day 5 (n = 5 per day).

Effects of the combined treatment on GIXA (in comparison with group 2, see Fig 3): Both GIXA-IgM and GIXA-IgG are strongly reduced until day 90.

NXA/GIXA Suppression by Repeated Xenoantigen and CY (Group 4)

To test whether the above observed NXA and GIXA reduction could be intensified to "suppression," the combined treatment, AG plus CY, was repeated on day 30 (n = 5). The result of this experiment is documented in Fig 5 and can be summarized as follows. (a) Effects of the repeated combined treatment on NXA (in comparison with group 1, see Fig 2): NXA-IgM are suppressed beyond day

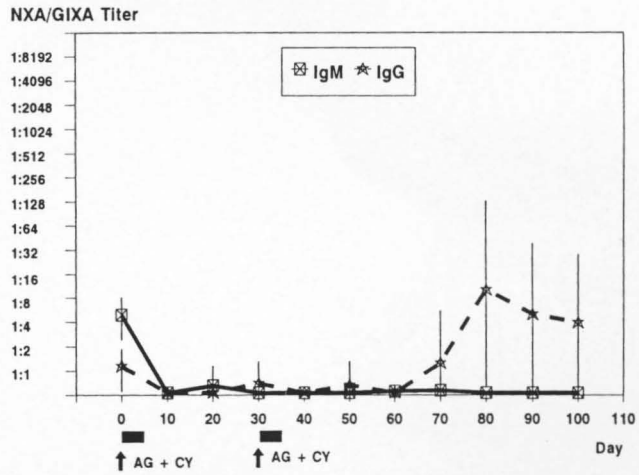

Fig 5. NXA/GIXA suppression by repeated xenoantigen and CY (group 4). In this group the combined treatment was repeated on day 30 (n = 5 per day).

100, NXA-IgG are suppressed until day 60. (b) Effects of the repeated combined treatment on GIXA (in comparison with group 2, see Fig 3): GIXA-IgM are suppressed beyond day 100. GIXA-IgG are suppressed until day 60, and are strongly reduced beyond day 100.

Specificity Control

To test whether the suppressive effects obtained by combined and repeated combined treatment are xenoantigen specific, rat sera from experimental group 3 (collected on day 30) and from group 4 (collected on day 60) were tested comparatively on human, porcine, and fish (trout) PBL target cells (n = 5). NXA/GIXA positive sera of experimental group 2 served as positive control. The result is not documented but can be summarized as follows: (a) Specificity control with porcine target cells: antibody binding on porcine target cells was similarly strongly suppressed as on human target cells. It is suggested that this nonspecificity with regard to two different mammalian species was due to a crossreactivity of GIXA on human and porcine PBL target epitopes. (b) Specificity control with fish target cells: in contrast to the above finding, antibody binding on fish PBL was not suppressed, thus indicating specificity of immunosuppression. This experiment clearly documents that the combined treatment is capable of inducing xenoantigen-specific B-lymphocyte tolerance.

CONCLUSIONS

Our data indicate that a time-limited combined treatment consisting of xenoantigen and CY, and in particular the repetition of this treatment protocol, appears to be a very effective new approach to downregulate the antibody-mediated primary xenograft rejection. This holds not only for GIXA but also for NXA, which are responsible for mediating hyperacute graft rejection. Unlike CY alone, the combination of both parameters, AG plus CY, induces long-lasting specific unresponsiveness, that is, tolerance against the sensitizing xenoantigen. The advantage of this approach with regard to a possible clinical application may be that CY is applied in a time-limited fashion rather than given permanently.

ACKNOWLEDGMENTS

The authors would like to thank Mrs K. Dohm, Ms C. Gier, Ms G. Prien, Mrs H. Steffen, and Mrs I. Wertz-Steinke for excellent technical assistance and Dr Rolf Siewing for kindly providing us with trout PBL.

REFERENCES

1. Milgrom F: In Hardy MA (ed). Xenograft 25. Amsterdam: Excerpta Medica, 1989, p 149

2. Herrlinger JD, Müller-Ruchholtz W: Z Immun Forsch 146: 195, 1973

3. Schroeder JO, Euler HH: Adv Exp Med Biol 260:203, 1989