Down-Regulation of Xenophile Antibodies by 15-Deoxyspergualin in an Experimental Animal Model

K. Ulrichs, H. Wang, and W. Müller-Ruchholtz

THE SUCCESS of clinical allogeneic organ transplantation has led to a shortage of human organ donors. A solution to this increasing problem is the use of donor organs from outside the human species. Since nonhuman primates are not available in sufficient numbers, are too expensive to breed and keep, and their use is limited by ethical concern, they are very unlikely candidates for xenotransplantation. Animals that are readily available in sufficient numbers, however, are cattle and pigs. Although it is a discordant donor species, the pig has been used as a donor species in initial clinical trials of fetal pancreatic islet transplantation.[1] Successful clinical xenotransplantation is limited not least by pre-existing natural antibodies (NXA) in the serum of the human recipient and by xenophile antibodies (XA), which are induced by the porcine xenograft. The solution to these humoral problems requires a threefold experimental approach: (a) a careful antibody analysis in the potential xenograft recipient; (b) the elimination of NXA from the recipient's serum before transplantation; and (c) the down-regulation of xenosensitization-dependent XA. This report continues and extends previous attempts from our laboratory to analyze and down-regulate these antibodies.[2–4]

It deals with the manipulation of xenophile antibodies in the experimental model "rat-antihuman" using 15-deoxyspergualin (15-DOS) and compares the effects with those obtained with leflunomide (LF) and cyclophosphamide (CY). 15-DOS was chosen for this study because there were preliminary reports indicating its efficacy also on B-lymphocyte reactivity[5] after our laboratory, among others, had shown that 15-DOS was not only well-tolerated, but was highly efficient in suppressing cell-mediated alloreactivity in the rat.[6]

MATERIALS AND METHODS

Animals

Lewis rats (3-month-old females, MHC:*RT-1*[1], specific pathogen-free; obtained from the Institut für Versuchstierzucht, Hannover, Germany, and kept under conventional conditions at the Institute of Immunology for the duration of the treatment and observation period) received 5×10^6 human peripheral blood lymphocytes intraperitoneally (IP) as xenogeneic antigen on day 0. 15-DOS was applied IP at a dosage of 2.5 mg/kg for 14 days, from day 0 until day +13; CY at a dosage of 20 mg/kg for 6 days, from day 0 until day +5; and LF at a dosage of 10 mg/kg for 10 days, from day 0 until day +9. The dosage of each drug was chosen individually depending on its effectiveness at B-lymphocyte suppression[7] or prolongation of organ allografts in the rat system.[6]

Experimental Design

The experimental design consisted of two treatment protocols: (a) treatment of nonsensitized LEW rats with 15-DOS to affect NXA; and (b) treatment of xenogeneically sensitized LEW rats (a single injection of human PBL on day 0 in combination with either 15-DOS, LF, or CY) to affect sensitization-dependent XA. Drug treatment always started on day 0. Each protocol included an untreated control group of rats. Tail vein blood was collected at 10-day intervals for analysis of NXA and XA by standard flow cytometry.

Determination of NXA and XA in Indirect Immunofluorescence

Similar to previous studies,[3,4] rat sera were diluted 1:2, 1:4, 1:8, etc, to determine IgM and IgG antibody titers in indirect immunofluorescence (FACS) using human PBL as target cells. Secondary antibodies were: (a) fluorescein (DTAF)-conjugated goat antirat IgG, Fc fragment specific (Dianova, Hamburg, Germany); and (b) fluorescein (DTAF)-conjugated $F(ab')_2$ fragment goat antirat IgM, μ-chain specific (Dianova). The titer was determined by the highest serum dilution at which 100% antibody binding was observed above background fluorescence. When binding occurred beyond titer 1, ie, in the range of 0% to 100%, the antibody content was determined indirectly by the percentage of fluorescent target PBL.

RESULTS

Influence of 15-DOS on NXA

To analyze whether 15-DOS is effective in down-regulating NXA in nonsensitized LEW rats, the drug was applied from day 0 until day +13 at a dosage of 2.5 mg/kg per day. The results of this treatment are shown in Fig 1 for both IgG (Fig 1A) and IgM (Fig 1B) and can be summarized as follows:

1. LEW rats, which were bred and raised under specific pathogen-free (SPF) conditions, but were then kept under conventional conditions for the duration of the treatment and observation period of about 100 days, increase their production of NXA-IgG antibodies constantly. This can be seen from the increasing

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, Brunswikerstr 4, D-24105 Kiel, Germany.

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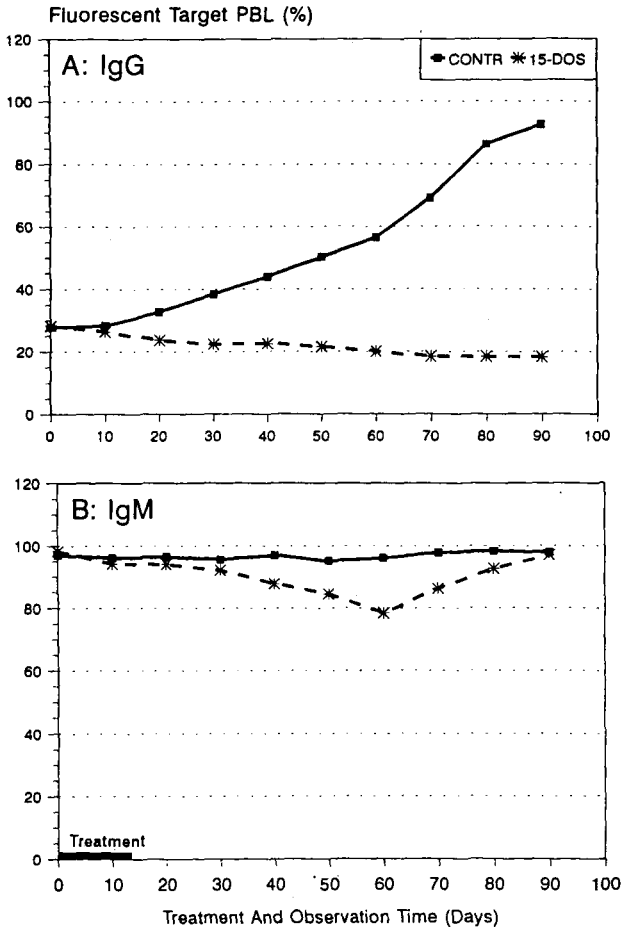

Fig 1. Effects of 15-deoxyspergualin (15-DOS) on the production of natural xenophile antibodies (NXA) in nonsensitized, normal LEW rats, which were bred and raised under SPF conditions, but kept under conventional stabling conditions for the time of treatment and observation. LEW rats received 2.5 mg/kg per day 15-DOS from day 0 until day +13 or remained untreated (CONTR). NXA in the rat serum were determined by indirect immunofluorescence (FACS) using human PBL as target cells in a binding assay. **(A)** Production of NXA-IgG; **(B)** production of NXA-IgM (n = 5 per group).

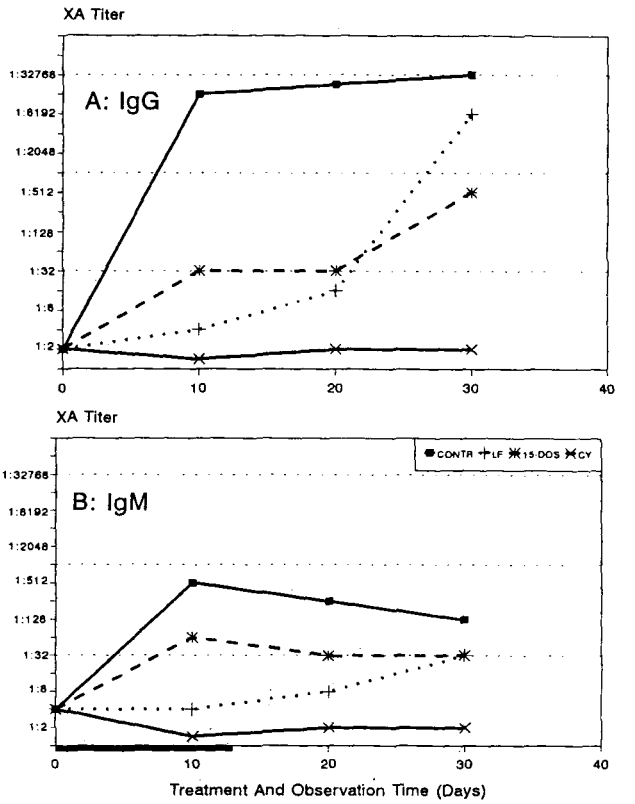

Fig 2. Effects of 15-deoxyspergualin (15-DOS), leflunomide (LF), and cyclophosphamide (CY) on xenosensitization-dependent antibodies (XA) in LEW rats, which were bred, raised, and kept under conventional stabling conditions. The rats received a combined treatment, consisting of xenoantigen (human PBL) on day 0 and a limited course of each drug (15-DOS, 2.5 mg/kg per day for 14 days; or LF, 10 mg/kg per day for 10 days; or CY, 20 mg/kg per day for 6 days; treatment start was always day 0). Another group of rats remained untreated (CONTR). **(A)** Production of XA-IgG; **(B)** production of XA-IgM (n = 5 per group).

percentage of human PBL target cells reacting with the serum NXA. The increase, which is very likely due to sudden microbial contamination under the new stabling conditions, is limited to the isotype IgG and was never seen with NXA-IgM.

2. 15-DOS, applied for 14 days, significantly inhibits the NXA-IgG production increase up to day 90, but it is incapable of effectively down-regulating NXA beyond background reactivity (untreated control day 0).
3. This does not hold for NXA-IgM, which (a) do not increase following the change in stabling conditions, and (b) remain unaffected by the drug.

Reduction of NXA and XA by Xenoantigen Plus Drug: A Comparative Study Using Different Drugs

To test the effects of the drugs on antibodies (XA) in sensitized animals, a combination treatment, consisting of xenoantigen on day 0 and a limited period of drug application, was performed using either 15-DOS, LF, or CY. The drugs were applied under comparative experimental conditions and were tested in indirect immunofluorescence (FACS). Fig 2 shows the results for IgG (Fig 2A) and for IgM antibodies (Fig 2B). The main findings are as follows: (a) antibody production switches from IgM to IgG isotype after xenogeneic sensitization on day 0 (compare titers of untreated control animals on day 0 with titers on day 10); (b) of the three drugs, CY is the most effective at down-regulating XA to or even below background level (IgG and IgM titers on day 0); and (c) as to the effects of the individual drugs, the strong to moderate effect of LF is reversible (see day 30, which is 20 days after termination of

LF application). The moderate effect of 15-DOS, however, lasts until day 90 (data not shown), whereas the strong effect of CY lasts until day 70 (data not shown).

DISCUSSION

As documented in Fig 1, 15-DOS has no effect on NXA, which were generated in LEW rats under SPF conditions. However, 15-DOS develops a significant suppressive effect on the up-regulation of NXA-IgG antibodies when these rats come under normal microbial exposure. The pool of natural xenophile antibodies may consist of a mixture of antibodies; eg, a product of endogenous immune network regulation ("germ line determined"[8]) and a product of microbial sensitization in postnatal life.

Our data indicate that a time-limited combined treatment, consisting of a single injection of xenoantigen and a time-related drug application, is an effective experimental approach to down-regulate the xenograft-dependent humoral immune reactivity, which, in addition to preformed natural antibodies (NXA), mediates acute xenograft rejection. Of the three drugs tested under comparative conditions, the alkylating agent, CY, proved to be the most powerful one. This was not unexpected, since CY has been known for a long time to be one of the most effective drugs with immunosuppressive potential toward B lymphocytes. However, because of its side effects clinicians hesitate to use it in patients unless they have to treat severe cases, eg, severe forms of systemic lupus erythematosus.[9,10] This disadvantage necessitates a continuous search for similarly effective drugs, but with fewer side effects. Therefore, 15-DOS and LF were tested. In the dosages used, neither of the two substances showed significant side effects in rats. Keeping this important point of concern in mind, their comparatively moderate immunosuppressive effect, which admittedly was somewhat disappointing, should nevertheless be regarded valuable for the manipulation of xenophile antibodies. To our knowledge this is one of the few reports indicating that 15-DOS not only successfully inhibits cell-mediated allograft rejection, which was also extensively studied in our laboratory,[6] but that it is also effective in down-regulating B-lymphocyte reactivity.[9] This leads to growing interest in xenogeneic transplantation.[11,12] CY, 15-DOS, and the isoxazole derivative, LF, taken together, provide an interesting opportunity to combine immunobiological effectivity of various drugs at well-tolerable dosages over limited periods of time.

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