Xenotransplantation 2001: 8: 15–23
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XENOTRANSPLANTATION
ISSN 0908–665X

Removal of bowel aerobic gram-negative bacteria is more effective than immunosuppression with cyclophosphamide and steroids to decrease natural α-Galactosyl IgG antibodies

Mañez R, Blanco FJ, Díaz I, Centeno A, Lopez-Pelaez E, Hermida M, Davies HFS, Katopodis A. Removal of bowel aerobic gram-negative bacteria is more effective than immunosuppression with cyclophosphamide and steroids to decrease nautral α-Galactosyl antibodies. Xenotransplantation 2001; 8: 15–23. © Munksgaard, Copenhagen

Abstract: Natural α-Galactosyl (Gal) antibodies play an important role in the rejection of pig xenografts by humans and Old World monkeys. In this study we investigate the efficacy of two different strategies to reduce the serum level of natural anti-Gal antibodies. On the one hand, removal of aerobic gram-negative bacteria from the intestinal flora, because anti-Gal antibodies appear to be produced as a result of the continuous sensitization by these microorganisms. On the other hand, we studied the effect on these antibodies of an immunosuppressive regimen of cyclophosphamide and steroids. Ten baboons were treated for three months with norfloxacin (Nor Group; n=6) or cyclophosphamide and steroids (CyP Group; n=4). A further four baboons did not receive any treatment (Control Group). Aerobic gram-negative bacteria became negative in stools of the Nor Group after two weeks of treatment, and remained undetectable until week 7. Thereafter, a gradual increase on the fecal concentration of aerobic gram-negative bacteria was observed despite the norfloxacin treatment. The mean anti-Gal IgG in the Nor Group gradually declined from week 4 to 9 to a mean of $62.7 \pm 18\%$ of the baseline level, and during this period were significantly lower than in the CyP ($P<0.02$) and the Control ($P<0.05$) groups. No differences were observed between the three groups during the 16 weeks of follow-up in serum levels of anti-Gal IgM, hemolytic anti-pig antibodies, total IgG, IgM and IgA. In conclusion, removal of normal aerobic gram-negative bacteria from the intestinal flora is more effective than immunosuppression with CyP and steroids in reducing the level of natural anti-Gal antibodies, although there is no discernible effect on IgM antibodies.

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Key words: baboon – natural antibodies – rejection – selective intestinal decontamination – xenotransplantation

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Received 26 April 2000;
Accepted 27 July 2000

Introduction

Xenotransplantation of pig organs into humans, apes and Old World monkeys has been precluded so far by severe immunological reactions that lead to a prompt xenograft failure. Hyperacute rejection is the earliest type of rejection, beginning immediately upon reperfusion [1]. This reaction is mediated by xenoreactive antibodies present in the recipient at the time of transplantation, which bind xenograft endothelial cells and activate the complement system [2,3]. For many years this rejection was the most important barrier for the success of xenotransplantation of pig organs in Old World monkeys [4]. Recently, several methods have been developed to elude hyperacute rejection. These include transient depletion of

xenoreactive natural antibodies [4], inhibition of complement by soluble factors [4], and particularly the use of organs from pigs transgenic for human complement regulatory proteins [5–7]. However, when hyperacute rejection is averted, other forms of rejection emerge, destroying the xenograft within days to a few weeks after transplantation [7]. The most prevalent cause of failure in these cases is an acute vascular rejection, which appears to be mainly caused by xenoreactive antibodies [7], although NK cells and macrophages may also be involved [8]. Therefore, xenoreactive antibodies have a primary role not only in hyperacute rejection but also in acute vascular rejection of pig organs transplanted in Old World monkeys.

Xenoreactive antibodies are either natural, also named pre-formed, because they are present at the time of transplantation without a known history of sensitization, or elicited, which are those antibodies produced by xenograft stimulation. Although both natural and elicited antibodies may recognize the same antigens, they appear to be produced by different immunological mechanisms. Natural antibodies are generated by a T-cell independent response, whereas the production of elicited antibodies is T-cell dependent [9]. The xenoreactive antibodies responsible for hyperacute rejection of pig xenografts transplanted into humans, apes and Old World monkeys are natural antibodies. These are mainly IgM and their major target is the α-Galactosyl (Gal) epitope [10–12], present in non-primate mammals, prosimians and New World monkeys, all of which lack anti-Gal antibodies [13]. In acute vascular rejection it is unclear whether the xenoreactive antibodies involved are natural, elicited or both. However, humans and baboons exposed in vivo or ex vivo to pig organs or cells are clearly stimulated to produce anti-Gal IgM and IgG [14–16]. The maintenance of the main specificity for xenoantibodies before and after the exposure to pig antigens suggests that natural xenoantibodies remain important in the immunological reactions mediated by these antibodies after transplantation.

Removal or neutralization of natural anti-Gal antibodies has been achieved so far by several methods. These include non-specific techniques such as plasmapheresis, xenogenic organ perfusion or infusion of monoclonal anti-idiotypic antibodies, and specific techniques such as immunoadsorption with αGal columns or perfusion with soluble αGal sugars [4]. However, all them have the same problem. The effect on natural anti-Gal antibodies lasts only a few hours after the procedure ends, allowing only the prevention of hyperacute rejection. To obtain an impact on avoiding acute vascular rejection, a sustained reduction of anti-Gal antibodies is required. If natural antibodies do participate to some extent in this reaction, a sustained decrease in their levels is necessary. So far, most of the immunosuppression protocols used in pig to non-human primate xenotransplantation include cyclophosphamide (CyP) and steroids to inhibit antibody production [4–7]. These drugs have demonstrated clinical efficacy in several auto-immune diseases mediated by natural antibodies [17–20]. However, in the xenotransplantation of pig organs to non-human primates the only effect demonstrated so far is a reduction on the return rate of natural anti-Gal antibodies if they are previously depleted with αGal columns [21].

While natural anti-Gal antibodies function in the xenotransplantation setting to induce hyperacute rejection, its physiological role is in homeostasis. These antibodies bind to carbohydrate structures exposed by several aerobic gram-negative bacterial strains [22,23], and share an immunoglobulin (Ig) gene structure with antibodies to common infectious agents [24], suggesting that they may be involved in the defense against infectious micro-organisms. Anti-blood Group isohemagglutinins is another family of natural antibodies that appear to be produced as a result of constant immunization against intestinal flora [9,25]. The similarities between natural anti-Gal antibodies and anti-blood Group isohemagglutinins, along with the presence in normal intestinal flora of gram-negative bacterial strains that interact with the former antibodies lead to the hypothesis that these micro-organisms provide a constant antigenic stimulation for the synthesis of natural anti-Gal antibodies [23]. If this is true, elimination from the bowel of micro-organisms that exhibit Gal epitopes should remove the stimulus for production of natural anti-Gal antibodies, and thus to a sustained decrease in their levels.

In the present study, we compared the effect of two different strategies on the serum levels of natural anti-Gal antibodies in baboons. On the one hand, we removed aerobic gram-negative bacteria from the intestinal flora, using an oral non-absorbable or poorly absorbable antibiotic such as norflaxacin [26,27], which has demonstrated efficacy in producing a selective removal of these micro-organisms in healthy subjects [28], and in patients with granulocytopenia, cirrhosis or liver transplantation [27,29,30]. On the other hand, we tested the effect of an immunosuppression regimen of CyP and steroids.

Materials and methods

Animals and study groups

A total of 14 baboons *(Papio Anubis)*, purchased from Consort Bioservices (Steyming, UK), were used in this study. The animals, weighing 5 to 8 kg, were housed in a facility approved and accredited by the Galicia Department of Agriculture. The studies were approved and monitored by the Research Committee of Juan Canalejo Medical Center.

Six baboons received norfloxacin 100 mg/day orally for 3 months (Nor Group), whereas another four baboons were treated during the same period with cyclophosphamide (CyP) 500 to 1000 mg/m^2 i.v. every 5 to 7 days, to maintain a white blood cell (WBC) count of between 2000 and 3000 cells/mm^3 (CyP Group). The CyP Group animals also received during the first two weeks, three pulses of 15 mg/kg of methylprednisolone i.v., followed by 5 mg/kg three times a week of prednisolone tapered to a maintenance dose of 2 mg/kg three times a week over four weeks. A further four baboons did not receive any treatment (Control Group).

Samples

Weekly stool samples were obtained from baboons of the Control and Nor groups during the 4 months. Serum samples for assaying levels of anti-Gal IgG, IgM and hemolytic anti-pig antibodies were taken at weekly intervals from the three groups during the same period of time. One additional sample was obtained from the baboons of the Nor Group at week 20. Also, serum samples were obtained before any treatment and once a month for 4 months to study total IgG, IgM and IgA levels. Finally, blood samples were drawn every other day in the CyP Group to follow the WBC count.

Collection and processing of specimen for fecal studies

Stool samples were collected in non-sterile plastic containers and the specimen weight was recorded. Fairly solid specimens were homogenized in a stomacher; liquid samples were homogenized by vortexing. A sample of 0.5 g of fresh feces was diluted 1:100 in 0.9% NaCl with the use of a mixer. Then 0.1-ml aliquots of this dilution and further 100-fold dilutions (to 10^{-6}) were spread onto MacConkey agar (aerobic gram-negative) and blood agar with nalidixic plates (aerobic gram-positive) (Oxoid Ltd, Basingstoke, UK). Plates were incubated aerobically at 36 °C for 24 to 48 h. Different colony types were identified through gram staining and counted as the viable $\log_{10}$ per g (wet weight) of feces (colony-forming units). The lower limit of detection was 3.0 $\log_{10}$ colony-forming units (CFU) per g of feces.

Measurement of anti-Galα1–3Gal antibodies by ELISA

Microtiter plates were coated with 50 μg/well αGal(1–3)βGal(1–4)GlcNAc linked to human serum albumin (HSA) (Dextra Labs, Reading, UK) (5 μg/ml) in 0.1 m carbonate buffer pH 9.6 overnight at 4 °C. Coated plates were blocked with 200 μl/well of 0.5% Tween/PBS for 1 h, at room temperature. After washing, duplicate dilutions of baboon serum prepared with 0.5% Tween/PBS, ranging from 1:2.5 to 1:320 or 1:10 to 1:1280, were pipetted (50 μl/well) to the antigen-coated wells and into uncoated, blank wells serving as controls for non-specific antibody binding. After an incubation of 1 h at room temperature, plates were washed six times with 0.1% Tween/PBS and incubated with horseradish peroxidase-labeled goat F(ab′)2 anti-human IgG (gamma chain specific) or IgM (mu chain specific)-(Sigma, St Louis, MO, USA) at 1:200 in 0.5% Tween/PBS, for 1 h at room temperature. After six washes with 0.1% Tween/PBS, the plate was developed with a solution of orthophenylene diamine (OPD, Sigma) prepared according to the manufacturer's instructions and quenched with 1 m H_2SO_4 before reading absorbances at 492 nm. The levels of anti-Gal IgG or IgM in different assays were normalized by comparison with a pool of 50 human sera provided by the local Blood Bank, which was included in all the assays

Measurement of hemolytic anti-pig antibody titer

Baboon clotted blood samples were spun for 5 min at 3000 r.p.m. at 4 °C. Sera were then collected, transferred into Eppendorf tubes, and heat inactivated at 56 °C for 30 min. Subsequently, each serum was serially diluted in a 96-well plate with complement fixation diluent (ICN, Costa Mesa, CA, USA), from 1:2.5 to 1:1280. After the addition of rabbit complement (Serotec, Oxford, UK) and 1% porcine red blood cells, the plates were placed in an orbital incubator at 37 °C for 1 h. The plates were then centrifuged for 10 min, and 100 μl of supernatant from each well were transferred into reading plates. The reading was performed by a Labsystems Multiskan Plus (Labsystems, Helsinki, Finland) at a wavelength of 420 nm. The mean absorbance of the two replicates was calculated for each dilution and expressed in area under the curve (AUC) units, considering the number of AUC units of the pool of human control serum as equal to 1.

Table 1. Baseline level of anti-Gal IgM, IgG and hemolytic anti-pig antibodies

Baboon	Group	Anti-Gal IgM*	Anti-Gal IgG*	Hemolytic anti-pig†
1	Nor	0.62	0.69	1.35
2	Nor	0.69	0.29	0.95
3	Nor	0.90	0.93	1.20
4	Nor	0.72	0.62	0.93
5	Nor	0.79	0.77	1.09
6	Nor	0.83	0.34	0.90
7	Control	0.72	0.71	1.10
8	Control	0.71	0.93	1.24
9	Control	1.06	0.86	1.28
10	Control	0.45	0.39	0.8
11	CyP	0.79	0.56	1.12
12	CyP	0.78	0.72	1.37
13	CyP	0.55	0.36	0.68
14	CyP	0.65	0.59	1.21

*Normalized by comparison with a pool of human sera (pool human sera = 1).
†The values represent AUC units in comparison with the pool of human sera (pool human sera = 1 AUC unit).

Pretreatment serum level of anti-Gal antibodies and hemolytic anti-pig antibody titer

In baboons, as in humans, the serum level of anti-Gal IgG and IgM antibodies, and the serum hemolytic anti-pig antibody titer, show large variations between individuals [31]. This precludes the use of average antibody titers to evaluate changes in a group of several baboons. The use of a shift from pretreatment level for each individual baboon is necessary to give homogeneity to the results.

Table 1 shows the pretreatment level of anti-Gal IgG, IgM and hemolytic anti-pig antibody titer of the 14 baboons. The weekly values obtained from every baboon are expressed as a percentage of the baseline level. To summarize and compare the data from the three groups, the mean $\pm$ S.D. shift from the individual baseline levels of the baboons in each group was estimated every week.

Statistical analysis

ANOVA for repeated measures was used to compare the level of anti-Gal IgM, IgG and hemolytic anti-pig antibodies between the three study groups.

Measurement of total IgM, IgG and IgA levels

The concentrations of total IgM, IgG and IgA were determined by nephelometry using a BN II nephelometer (Behring, Marburg, Germany).

Results

Effect of norfloxacin on fecal flora

As illustrated in Fig. 1, the mean counts of aerobic gram-negative bacteria in the stools before treatment were 5.37 ± 5.0 and 5.30 ± 5.18 ($\log_{10}$ CFU)/g in the Nor and Control Groups, respectively. One week after norfloxacin treatment was started, the mean count of aerobic gram-negative bacteria declined to 3.98 ± 3.0 ($\log_{10}$ CFU)/g in the Nor Group. Between weeks 2 and 7, no aerobic gram-negative bacteria were found in the feces. At week 8, a mean count of 3.18 ± 3.0 ($\log_{10}$ CFU)/g of aerobic gram-negative bacteria was detected in the stool, despite the norfloxacin treatment. Thereafter, a gradual increase of the fecal concentration of aerobic gram-negative bacteria occurred in the Nor Group. At week 12, within three weeks of the cessation of the norfloxacin treatment, the counts of aerobic gram-negative bacteria returned to pretreatment

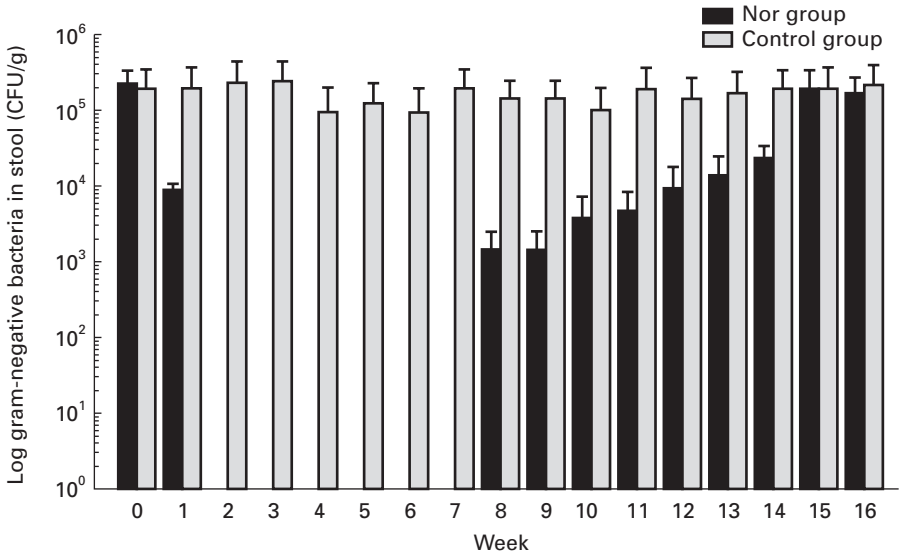

Fig. 1. Mean weekly counts of aerobic gram-negative bacteria in stools from the Nor and Control Groups.

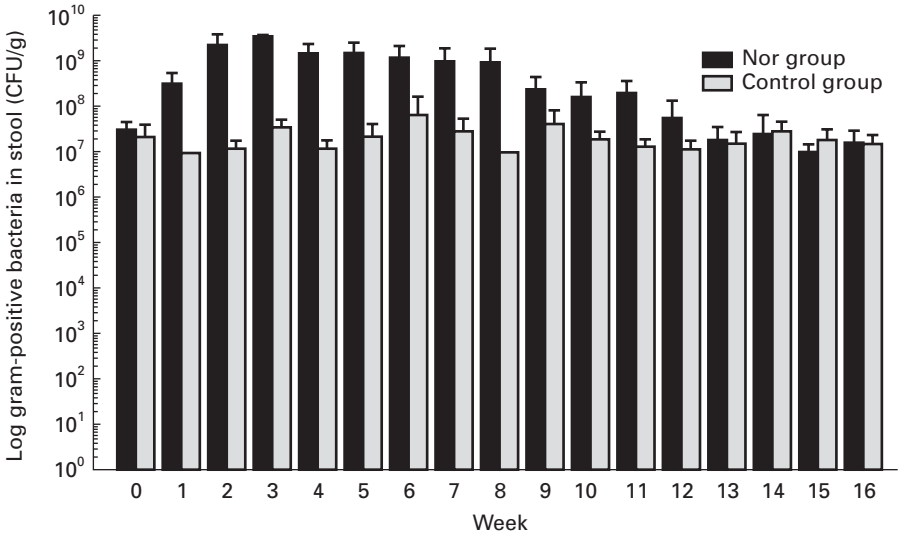

Fig. 2. Mean weekly counts of aerobic gram-positive bacteria in stools from the Nor and Control Groups.

levels. During the same period, no change was observed in the mean counts of aerobic gram-negative bacteria in the Control Group.

At the beginning of the study, the mean baseline counts of aerobic gram-positive bacteria in the feces were 7.53 ± 7.07 and 7.35 ± 7.28 ($\log_{10}$ CFU)/g in the Nor and Control Groups, respectively. In the Nor Group, the mean count gradually increased to a maximum mean level of 9.56 ± 8.08 ($\log_{10}$ CFU)/g at week 3, remaining above 9 $\log_{10}$ CFU/g until week 7, whilst no change was observed in the Control Group during the 16 weeks of the study period (Fig. 2).

Effect of norfloxacin on serum anti-Gal levels

Figure 3(A) shows the levels of anti-Gal IgG antibodies in the Nor and Control Groups. During the first three weeks, no differences were found between the two groups. At week 4, anti-Gal IgG levels dropped to give a mean of $74.3 \pm 15.5\%$ from the pretreatment levels in the Nor Group. Thereafter, anti-Gal IgG gradually declined to a minimum mean of $62.7 \pm 18\%$ of the pretreatment level at week 9. Between weeks 5 and 9, the mean anti-Gal IgG level in the Nor Group was significantly lower than in the Control Group ($P<0.05$). At week 10, two weeks after aerobic gram-negative bacteria returned to the stool, anti-Gal IgG levels started a progressive increase to $86.5 \pm 15\%$ of the pretreatment level at week 16 and reached the baseline pretreatment level at week 20.

The mean levels of anti-Gal IgM antibody in the Nor and Control Groups are shown in Fig. 3(B). No differences were found between these two groups in any of the 16 weeks of study period.

Effect of cyclophosphamide on serum anti-Gal levels

Baboons treated with CyP received an average daily dose of 7, 6.6, 6.9 and 5.9 mg/kg, respectively. Figure 4(A) shows the mean levels of anti-Gal IgG in the CyP and Control groups over the 16 weeks of the study period. No differences were observed between these groups at any time. However, the mean levels of anti-Gal IgG were significantly higher in the CyP Group than in the Nor Group between weeks 4 and 9 ($P<0.02$).

The mean levels of anti-Gal IgM in the CyP and Control Groups are illustrated in Fig. 4(B). There were no differences between these two groups, or with the Nor group, in any week of the follow-up.

Effect of norfloxacin and cyclophosphamide on the level of hemolytic anti-pig antibodies

Figure 5(A) shows the mean levels of hemolytic anti-pig antibodies in the Nor and Control Groups. No differences were observed at any time between these two groups. Also, the mean levels of hemolytic anti-pig antibody titer from the CyP Group were similar to the Control Group during the 16 weeks of the study (Fig. 5B).

Effect of norfloxacin and cyclophosphamide on serum total IgG, IgM and IgA

To investigate whether norfloxacin or CyP have any effect on the total level of antibodies, total IgG, IgM and IgA were measured before, during and after the treatments. Table 2 shows the mean $\pm$ S.D. of these immunoglobulins in the three groups. No changes were observed on the total level of any class of immunoglobulin before, during or after treatments with norfloxacin or CyP and steroids. Also, the level of IgG, IgM and

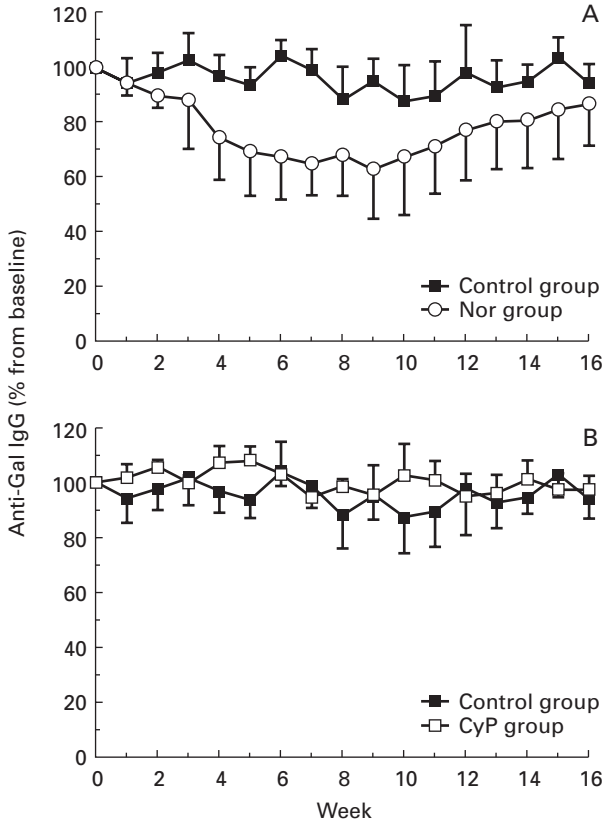

Fig. 3. Mean weekly anti-Gal IgG (A) and IgM (B) in the Nor and Control Groups. The values represent the percentage of the pretreatment level.

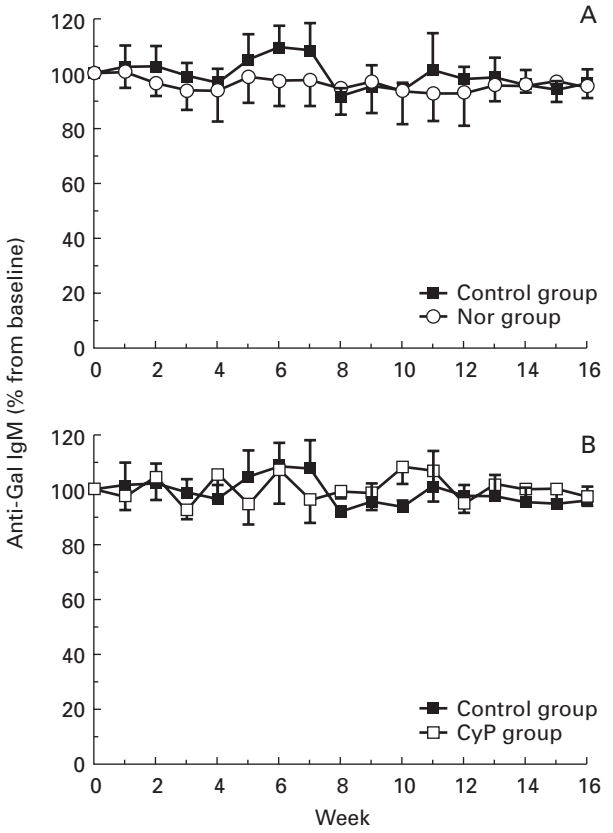

Fig. 4. Mean weekly anti-Gal IgG (A) and IgM (B) in the CyP and Control Groups. The values represent the percentage of the pretreatment level.

IgA were similar in the Nor, CyP and Control Groups.

Discussion

The present study demonstrates that removal of intestinal aerobic gram-negative bacteria from the bowel with a non-absorbable antibiotic such as norfloxacin is associated with a decrease in the serum level of natural anti-Gal IgG antibody. After two weeks of norfloxacin treatment aerobic gram-negative bacteria became undetectable in the fecal flora. The decline of natural anti-Gal IgG was significant after week 4, with the lowest level detected at week 9 of norfloxacin treatment, one week after aerobic gram-negative bacteria returned to the stool. The two-week delay observed between the absence of aerobic gram-negative bacteria and the lowest level of natural anti-Gal IgG levels may be due to the relatively long half-life of IgG antibody, which is around 23 days.

Elimination of aerobic gram-negative bacteria from intestine was more effective than immunosuppression in reducing the serum level of natural anti-Gal IgG antibodies. However, the effect was incomplete, because the lower level obtained was 63% of the baseline level. Several mechanisms may explain the effect observed on natural anti-Gal IgG. Norfloxacin could have a direct effect over some transcriptional activator factors of B cells, because it inhibits immunoglobulin production [32]. However, suppression of production of anti-Gal IgG by plasma cells or memory cells by norfloxacin should be specific for this antibody because in this study the total level of immunoglobulins did not change with the treatment. Another explanation may be the decrease of serum natural anti-Gal IgG antibodies as a consequence of the elimination of aerobic gram-negative bacteria from the intestinal flora. In this case, a decrease in antigen stimulation may reduce the production of anti-Gal IgG by plasma cells or may lead to a failure of isotype Ig switching by affecting factors such as gram-negative lypopolysaccharides (LPS) that play a critical role in this process [33].

The incomplete effect observed on natural anti-Gal IgG, and the lack of effect on natural anti-Gal IgM suggests the production of these antibodies, to some extent in the former and completely in the latter, through mechanisms independent of intest-

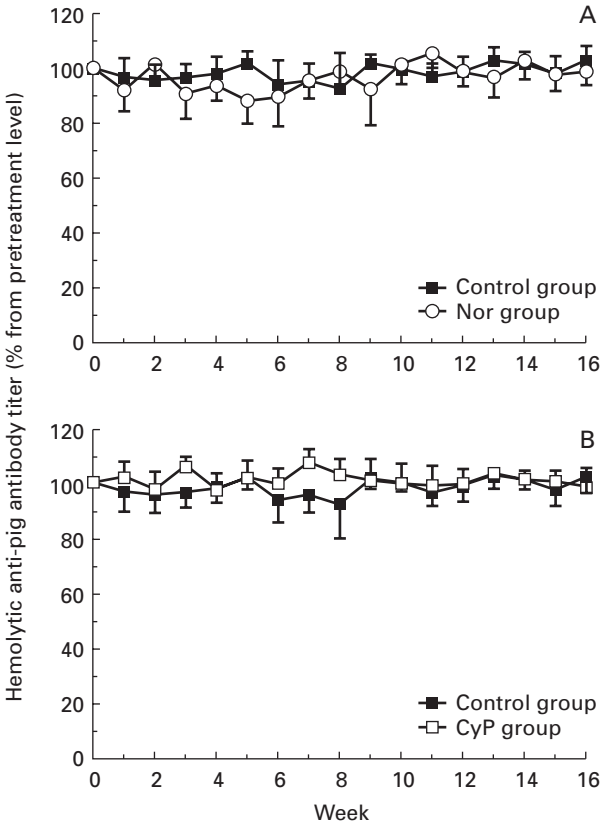

Fig. 5. Mean weekly hemolytic antibody titer in the Nor (A) and CyP (B) Groups. The values represent the percentage of the pretreatment level.

inal bacteria stimulation or by micro-organisms other than aerobic gram-negative bacteria. Gal epitopes have been described in the carbohydrate portion of LPS from gram-negative bacteria [23], in parasites and in retroviral envelopes [34,35]. Of these, only gram-negative bacteria are present in physiological conditions in humans. In baboons, we cannot rule out the fact that parasites also play a role in the production of anti-Gal IgG and IgM antibodies, because detection of these micro-organisms in stools is associated with higher level of antibodies [36]. However, the 14 animals included in this study were totally free of parasites and therefore similar to the human situation.

The oral non-absorbable antibiotic used in the study was only active against aerobic gram-negative bacteria. It cannot be ruled out that a broader spectrum of antibiotic therapy might have a major impact on anti-Gal antibodies. However, the lack of evidence that other micro-organisms from normal intestinal flora carry the Gal antigen besides aerobic gram-negative bacteria, along with the risk of rising resistant micro-organisms with a broad-spectrum treatment, lead us to choose a single antibiotic treatment. Norfloxacin has proven clinical efficacy on the prevention of gram-negative infections in cirrhotic and transplant patients [29,30]. Despite this, it was associated with an augmentation of aerobic gram-positive, and the total elimination of aerobic gram-negative micro-organisms for only 6 weeks. Thereafter, the isolation of aerobic gram-negative bacilli from stools, despite the maintenance of the antibiotic treatment, suggests the appearance of norfloxacin resistant micro-organisms.

The immunosuppressive regimen used in this study, which included CyP and steroids, did not have any effect on the level of natural anti-Gal IgG or IgM. Previous observations in humans showed that primary and secondary humoral immunodeficiencies, as well as active immunosuppression with steroids, are associated with a decline in natural anti-Gal IgG titers [37]. The lack of efficacy of CyP and steroids on natural anti-Gal antibody titer, despite using an average dose of CyP and steroids significantly higher than that recommended for immunosuppression in humans, suggests that baboon B cells may be more resistant than human B cells to these drugs or that the pharmacokinetics and bioavailability of the active CyP metabolite is different in baboons. This was corroborated in our study by the lack of effect observed with CyP and steroids on total IgG, IgM and IgA levels during the three months of treatment. When the same treatment is used for auto-immune diseases, clinical improvement is associated with a decline in the total level of IgM, IgG and IgA [19]. The results of this study also differ from the efficacy observed with high doses of CyP in prolonging xenograft survival in pig to non-human primate xenotransplantation [38]. This may indicate that the xeno-antibodies responsible for acute vascular rejection are mainly elicited instead of natural antibodies, and for this

Table 2. Total levels of immunoglobulins before, during and after norfloxacin and cyclophosphamide treatments

	IgG (mg/dL)*			IgM (mg/dL)*			IgA (mg/dL)*		
Group	Before	During	After	Before	During	After	Before	During	After
Nor	1020 ± 241	902 ± 176	855 ± 108	62 ± 32	75 ± 11	78 ± 15	25.3 ± 4.8	25.0 ± 1.7	25.9 ± 4.2
CyP	969 ± 214	834 ± 153	786 ± 186	74 ± 16	69 ± 26	82 ± 20	21.8 ± 6.6	30.5 ± 10.8	22.2 ± 7.1
Control	896 ± 231	795 ± 193	838 ± 97	68 ± 28	77 ± 18	71 ± 24	26.8 ± 2.9	27.1 ± 5.3	27.8 ± 4.6

*Mean ± S.D.

reason CyP is more effective. However, it should be noted that the average dose of CyP during the first 9 days of transplantation required to prolong pig xenograft survival is more than three times the average dose given in this study [38]. This dosage is associated with significant toxicity and when it was decreased to an average of twice the dose used in our protocol, all the xenografts underwent acute vascular rejection. Thus, it cannot be ruled out that keeping lower WBC counts through high CyP doses would have reduced the total level of Ig and particularly natural anti-Gal antibodies. Nevertheless, this type of treatment cannot be main-tained for very long and the objective of the present study was to obtain a sustained decrease of natural anti-Gal antibodies with a protocol that could be used clinically for long periods of time. In these circumstances the level of WBC must be maintained between 2000 and 3000 cells/mm^3 to avoid side-effects, particularly infectious complications.

In summary, removal of aerobic gram-negative bacteria from the bowel with norfloxacin is associated with a decrease in natural anti-Gal IgG. The intrinsic mechanisms responsible of this response remain to be elucidated. However, elimination of these micro-organisms with norfloxacin is the only method, besides the immunoadsorption techniques, that has been associated with a sustained reduction of at least some of natural anti-Gal antibodies.

Acknowledgments

We thank Dr Francisco Javier Muñiz for his assistance in the statistical analysis.

This work was funded in part by Novartis Pharma AG, Basel, Switzerland.

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