ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 7, pp. 862-866. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © N. V. Shilova, M. J. Navakouski, M. Huflejt, A. Kuehn, R. Grunow, O. Blixt, N. V. Bovin, 2011, published in Biokhimiya, 2011, Vol. 76, No. 7, pp. 1051-1056.

Changes in the Repertoire of Natural Antibodies Caused by Immunization with Bacterial Antigens

N. V. Shilova[1], M. J. Navakouski[1], M. Huflejt[2], A. Kuehn[3], R. Grunow[3], O. Blixt[4], and N. V. Bovin[1]*

[1]*Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (495) 330-5592; E-mail: bovin@carbohydrate.ru*
[2]*Department of Cardiothoracic Surgery, New York University School of Medicine, Bellevue Hospital, 462 First Avenue, Room 15N 20-22, 10016 New York, NY, USA; E-mail: margaret.huflejt@gmail.com*
[3]*Centre for Biological Safety (ZBS2), Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany; E-mail: grunowR@rki.de*
[4]*Institute of Cellular and Molecular Medicine, Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark; E-mail: olablixt@sund.ku.dk*

Received January 31, 2011
Revision received February 14, 2011

Abstract—The repertoire of natural anti-glycan antibodies in naive chickens and in chickens immunized with bacteria *Burkholderia mallei*, *Burkholderia pseudomallei*, and *Francisella tularensis* as well as with peptides from an outer membrane protein of *B. pseudomallei* was studied. A relatively restricted pattern of natural antibodies (first of all IgY against bacterial cell wall peptidoglycan fragments, L-Rha, and core N-acetyllactosamine) shrank and, moreover, the level of detectable antibodies decreased as a result of immunization.

DOI: 10.1134/S0006297911070170

Key words: bacterial polysaccharides, natural antibodies, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Francisella tularensis*

"Natural" refers to antibodies whose production does not require active antigenic stimulation. They are a part of the innate immunity and some of them can interact with host antigens, that is to say are autoantibodies [1-3]. These antibodies are produced by B-1 lymphocytes through a limited number of genes, which are not susceptible to somatic mutations, and belong predominantly to the IgM isotype. The most studied antibodies in human blood are those against the ABO system antigens, xeno-antigens, such as Galα1-3Galβ1-4GlcNAc, Forssman glycolipid GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc, and Hanganutsu–Diecher antigen Neu5Gcα2-3Galβ1-4Glc(NAc) as well as tumor-associated carbohydrate antigens Tn (GalNAcα), TF (Galβ1-3GalNAcα), and SiaTn (Neu5Acα2-6GalNAcα). Natural antibodies are involved in early host defense against viral, bacterial, and yeast infections prior to development of the strong adaptive immunity. They can participate in removal of apoptotic bodies and necrotic cells. Their regulatory functions such as prevention of autoimmune diseases, regulation of cell growth, hematopoiesis, etc. are also important. Only recently, a glycochip [4] has allowed collecting data on the repertoire of natural human anti-glycan antibodies [5].

Studies of human antibodies are the most important, but experimental models are necessary for elucidation of the genesis of natural antibodies. From them, one of the most convenient is a chicken model: a hen is immunized and antibodies, so called IgY, are isolated from a chicken egg [6]; therefore, in contrast to a mouse model, they are available in virtually unlimited amounts. The existence of natural anti-glycan antibodies in chicken has been shown in works [7-9], however, no detailed studies of IgY against carbohydrates have been carried out to date.

In the present work, chicken anti-glycan antibodies were studied using the same glycochip [4] as has been used earlier for studies of human natural antibodies. Natural antibody repertoires in chickens immunized with bacteria *Burkholderia mallei*, *B. pseudomallei*, and *Francisella tularensis* and in naive birds were compared.

Abbreviations: GMDP, N-acetylglucosaminyl-muramyldipeptide; OMP, outer membrane protein of *Burkholderia pseudomallei*.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Immunization. Chickens of White Leghorn breed were immunized with paraformaldehyde-inactivated bacteria three times. Birds denoted H32 and H61 were immunized with bacteria *B. mallei*; H33 and H60 with *B. pseudomallei*; and H62 with *F. tularensis*; birds denoted OMP1 and OMP2 were immunized with individual peptides (OMP-1 and OMP-2, respectively), which were fragments of a membrane protein isolated from *B. pseudomallei*. The peptides were from the division of Highly Pathogenic Microorganisms of the Centre for Biological Safety of the Robert Koch Institute (Berlin, Germany) and will be described in detail elsewhere. Immunization was performed intramuscularly as described [10]: first 10^6 and then twice 10^5 inactivated bacteria were injected; a 150 μg peptide dose injected first was followed by two doses of 100 μg. Eggs were collected 10 days after the last immunization [11]. Eggs of a control group (chickens H33 and H49) were collected prior to immunization.

IgY were isolated by precipitation with 3.5% aqueous solution of PEG-600 (Sigma-Aldrich, USA) as described [12].

Analysis on glycochip. The glycochips were printed as described [4] and contained ca. 200 carbohydrate ligands. The chips were treated for 15 min with 0.1 M phosphate buffered saline (PBS) (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4) (Sigma-Aldrich) containing 0.1% Tween-20 (ICN, USA) (PBS-0.1%). Then 50 μg/ml chicken antibody solution in PBS-0.1% containing 3% BSA was added and the mixture was shaken for 1 h under relative humidity of 80%. Then the chips were washed with PBS-0.05%, and solution of Alexa549-labeled antibodies against chicken IgY (Invitrogen, USA) diluted 1 : 100 with PBS-0.1% was added. Following the incubation on a shaker under relative humidity of 80% for 1 h, the chips were washed with PBS-0.05% and bidistilled water subsequently, and the fluorescence intensity was measured using a confocal ProScanArray HT scanner (PerkinElmer, USA) with 5 μm resolution (laser power 90%). The data were processed using ScanArray Express 3.0 software and the fixed 70-μm-diameter rings method as well as Microsoft Excel. Signals which fluorescence intensity was at least twice as high as the background value (i.e. that in glycan-free zones) were considered as significant.

RESULTS AND DISCUSSION

Studies of large cohorts of healthy donors [5] revealed the presence in human blood serum of antibodies that interact specifically with glycotopes typical of various bacteria, such as fragments of GlcNAc-containing peptidoglycans, rhamnosides, etc. These antibodies (IgG and IgM) may be classified as natural since they appear in nearly all donors and the observed variations in their levels are insignificant. It is generally accepted that formation of natural glycan antibodies is stimulated by normal intestinal flora [8]. Therefore, there is a risk of a "false positive" specific immune response to bacterial polysaccharides owing to the presence of pre-existing antibodies, some of which are detected in consistently high titers [5].

In the present work, the repertoire of pre-existing antibodies in naive chickens was studied using a carbohydrate microchip of >200 glycans [4] and open-access data of the Consortium for Functional Glycomics (CFG) [13], which were collected by means of a nearly identical chip. The repertoire of anti-glycan IgY in naive birds (Table 1) was found to be relatively limited: not more than 20 specificities were revealed compared to ~100 specificities in humans [5]. Close antibody profiles were observed for two birds (H33 and H49) of the same breed, which were from the same group and kept under the same conditions. Although the IgY profile reported by the CFG was different, common motifs could be seen.

Among common epitopes, first of all those typical of the repertoire of natural human antibodies should be noted. These are GMDP-Lys and chitooligosaccharides (i.e. repeating fragments of the bacterial cell wall peptidoglycan), L-rhamnose (a typical component of many O-polysaccharides), as well as lactosamine (Galβ1-4GlcNAc) and lactose (Galβ1-4Glc) motifs, which are characteristic both for lactobacteria and some other widespread bacteria and for core domains of carbohydrate chains of glycoproteins and glycolipids. In addition, antibodies were detected against L-fucose and fucosides, which may be common motifs in bacterial polysaccharides and mammalian glycans too. It is worth noting also binding of glycans typical of a particular bird only (e.g. mannopentaose in the sample H49; Table 1), which, apparently, reflects its individual immunological "history".

Then, groups of two, two and one birds were immunized with bacteria *B. mallei*, *B. pseudomallei*, and *F. tularensis*, respectively. For the animal H33, the data are available both before and after immunization. The appearance of antibodies against the immunizing bacteria was confirmed by enzyme immunoassay with the inactivated bacteria (data not shown). In this work, we were most interested in answering the questions: i) whether the auto-IgY that were typical of the naive birds can be detected in presence of species-specific immune (adaptive) antibodies, and ii) whether unspecific anti-glycan antibodies cross-reacting with glycans of glycoproteins or glycolipids appear as a result of immunization. Therefore, immunoglobulins (IgY) isolated from the immunized chickens were analyzed using the same glycochip as antibodies of the naive birds, and the results are shown in Table 2.

Noticeably, the average intensity of signals of antibodies after immunization was in general lower (e.g. the

Table 1. Carbohydrate specificity of antibodies of naive birds revealed by the glycochip

H33	H49	CFG data [13]
L-Rhaα	**L-Rhaα**	**L-Rhaα**
GMDP-Lys	**GMDP-Lys**	**GMDP-Lys**
Galβ4GalNAcβ3(Fucα2)Galβ4GlcNAcβ	**Galβ4GalNAcβ3(Fucα2)Galβ4GlcNAcβ**	**$(GlcNAcβ4)_3$**
$(GlcNAcβ4)_5$	**$(GlcNAcβ4)_5$**	Fucα2Galβ4Glcβ
$(GlcNAcβ4)_6$	**$(GlcNAcβ4)_6$**	Fucα2Galβ3GlcNAcβ
L-Fucα	**L-Fucα**	Fucα2Galβ4GlcNAcβ
$(Galβ4GlcNAc)_2$-3,6Galβ4GlcNAcβ	**$(Galβ4GlcNAc)_2$-3,6Galβ4GlcNAcβ**	Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ
GalNAcβ3Galα4Galβ4Glcβ	**GalNAcβ3Galα4Galβ4Glcβ**	Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3-Galβ4GlcNAcβ
Galα3Galβ4(Fucα3)GlcNAcβ	**Galα3Galβ4(Fucα3)GlcNAcβ**	GalNAcα4(Fucα2)Galβ4GlcNAcβ
$(Neu5Acα8)_2$Neu5Acα3(GalNAcβ4)-Galβ4Glcβ	**$(Neu5Acα8)_2$Neu5Acα3(GalNAcβ4)-Galβ4Glcβ**	Galα4(Fucα2)Galβ4GlcNAcβ
GalNAcα	Neu5Acα8Neu5Acα3GalNAcβ4Galβ4Glcβ	Galα6Glcβ
Galα3(Fucα2)Galβ3GalNAcα	Manα6(Manα3)Manα6(Manα3)Manβ	Galα4GlcNAcβ
GlcNAcβ4GlcNAcβ-Asn		Galα3(Galα4)Galβ4GlcNAcβ
ManNAcβ		Galα4Galβ4Glcβ
		Glcβ6Glcβ

Note: Structures recognized by IgY in all naive birds are shown in bold.

Table 2. Carbohydrate IgY pattern of immunized birds revealed by the glycochip

H32 *B. mallei*	H61 *B. mallei*	H33 *B. pseudomallei*	H60 *B. pseudomallei*	H62 *F. tularensis*
$(GlcNAcβ)_3$-3,4,6-GalNAcα	$(GlcNAcβ)_3$-3,4,6-GalNAcα	$(GlcNAcβ)_3$-3,4,6-GalNAcα	GlcNAcβ4Galβ4-GlcNAcβ	GlcNAcβ4Galβ4-GlcNAcβ
GalNAcα4(Fucα2)-Galβ4GlcNAcβ	GalNAcα4(Fucα2)-Galβ4GlcNAcβ	GalNAcα4(Fucα2)-Galβ4GlcNAcβ	Galα4(Fucα2)-Galβ4GlcNAc	GlcNAcβ4-$[HO_2C(CH_3)CH]$-3-O-GlcNAcβ
GMDP-Lys	GlcNAc-$[HO_2C(CH_3)CH]$-3-O-GlcNAcβ	Galβ4GalNAcα3-(Fucα2)Galβ4GlcNAcβ	Fucα2Galβ3GalNAcα	Galβ4GalNAcβ3(Fucα2)-Galβ4GlcNAcβ
			L-Rhaα	
			Galα4GlcNAcβ	
			Galα4Galβ4Glcβ	
			Galα4GlcNAcβ3-Galβ4GlcNAcβ	

immunization changed by an order of magnitude the average fluorescence value of a sample of antibodies from the animal H33) and their repertoire shrank; particularly, no antibodies to chitooligosaccharides and the lactose core were detected. Remarkably, at the same time in the samples H32, H61, and H33, antibodies appeared against an unusual $(GlcNAcβ)_3$-3,4,6-GalNAcα glycan, which was not detected in naive chickens (Table 1) and which

a

- -3)βDGlc*p*(1-3)αL6dTal*p*2Me(1-
- -3)βDGlc*p*(1-3)αL6dTal*p*2Ac(1-

b

- -3)βDGlc*p*(1-3)αL6dTal*p*(1-
- -3)βD6dmanHep*p*2Ac(1-
- -3)βDGlc*p*(1-3)αL6dTal*p*2Me4Ac(1-
- -3)βDGlc*p*(1-3)αL6dTal*p*2Ac(1-
- -3)βDGal*p*2Ac(1-4)αDGal*p*(1-3)βDGal*p*(1-5)βKdo*p*(2-

c

- αDGlc*p*(1-3)+
  |
  Subst(?-3)βDQui*p*NAc(1-4)+ |
  | |
  αDGal*p*NAc(1-2)βDMan*p*(1-4)+
  |
  βDGlc*p*(1-2)αDMan*p*(1-5)αKdo*p*(2-6)βDGlc*p*N(1-6)DGlcN
- -4)αDGal*p*NAcA6NH$_2$(1-4)αDGal*p*NAcA6NH$_2$(1-3)βDQui*p*NAc(1-2)βDQui*p*4NFo(1-
- αDGal*p*N(1-2)βDMan*p*(1-4)+
  |
  βDGlc*p*(1-2)αDMan*p*(1-5)αKdo*p*(2-6)βDGlc*p*N(1-6)DGlcN-ol
- αDGlc*p*(1-3)+
  |
  αDGal*p*N(1-2)βDMan*p*(1-4)+
  |
  βDGlc*p*(1-2)αDMan*p*(1-5)αKdo*p*(2-6)βDGlc*p*N(1-6)DGlcN-ol
- βDGlc*p*N(1-6)αDGlc*p*N(1-P
- -4)αDGal*p*NAcA6NH$_2$(1-4)αDGal*p*NAcA6NH$_2$(1-3)βDQui*p*NAc(1-2)βDQui4NFo(1-

Structures of the polysaccharides and lipopolysaccharides of *B. mallei*, *B. pseudomallei*, and *F. tularensis* (a-c, correspondingly) [14]. 6dmanHep, 6-deoxy-*manno*-heptose; 6dTal, 6-deoxytalose; 6dTal2Ac, 6dTal2Me, and 6dTal2Me4Ac are 2-O-acetyl-, 2-O-methyl-, and 2-O-methyl-4-O-acetyl-6-deoxytalose; GalNAcA6NH$_2$, 2-acetamido-2-deoxygalacturonamide; GlcN-ol, 2-amino-2-deoxyglucitol; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; QuiNAc, 2-acetamido-2-deoxyquinovose (2-acetamido-2,6-dideoxyglucose); Qui4NFo, 4-formamido-4,6-dideoxyglucose; Subst, O-polysaccharide substituent

occurs neither in bacterial polysaccharides nor in mammalian or avian glycans. These antibodies have been previously found in blood of healthy humans (P. S. Obukhova et al., unpublished data); their origin remains unknown. A comparison of the $(GlcNAc\beta)_3$-3,4,6-GalNAcα glycan structure with known sequences of polysaccharide immunogens (figure) [14] does not reveal any obvious structural similarity.

In addition to the whole bacteria, individual peptides from a *B. pseudomallei* outer membrane protein (OMP-1 and OMP-2) were used for immunization. In this case, no natural anti-glycan antibodies were detected in immunized birds at all, and IgY that were isolated as in the previous series of experiments bound on the glycochip only to the Galα1-4Galβ1-4Glcβ trisaccharide (P_k-antigen) at the average level of intensity (data not shown). Therefore, the immunization with both whole bacteria and individual bacterial peptides results in essentially the same results, namely, the pre-existing antibody titer decreases significantly and their repertoire shrinks, most likely owing to a competitive production of adaptive immunoglobulins. These findings are in contradiction to the generally accepted notion that the level of natural antibodies is little subjected to change even on strong antigenic stimulation [15]; therefore, further studies are necessary.

Even though the results are only preliminary owing to a small number of bacteria studied and a limited number of experimental animals, they enable conclusions important for certain practical implementations. Particularly, vaccination of humans with bacterial preparations (should the effect discovered reproduce in the case of the human immune system) may be accompanied by an attenuation of the natural humoral immunity. In addition, relatively close titers of immune and natural anti-carbohydrate antibodies (e.g. to L-Rhaα and the GM1 pentasaccharide, which was used as immunogen in GFG studies [13]) as well as well-known similarities between some bacterial antigens and mammalian and avian glycans may result in false interpretation of data on the specificity of the immune response to bacterial polysaccharides and lipopolysaccharides. Therefore, the information about the natural anti-glycan antibody repertoire in those animals that are usually used as models in studies of the immune response as well as on its externally influenced "plasticity" is not only desirable but also necessary.

We are grateful to M. L. Markelov and E. A. Pudova (Institute of Labor Medicine, Moscow, Russia) for the help with analyses on the glycochip.

This work was supported by grants from the Presidium of the Russian Academy of Sciences Program "Molecular and Cell Biology", the European Commission Marie Curie Program for support of the EuroGlycoArrays ITN project, the German Federal Ministry of Education and Research (project BiGRUDI), the Benzon Foundation, the Danish Research Council, the Danish Agency for Science, Technology and Innovation (FTP).

REFERENCES

1. Avrameas, S., and Ternynck, T. (1993) *Mol. Immunol.*, **30**, 1133-1142.
2. Avrameas, S. (1991) *Immunol. Today*, **12**, 154-159.
3. Dighiero, G., and Rose, N. R. (1999) *Immunol. Today*, **20**, 423-428.
4. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M. E., Alvarez, R., Bryan, M. C., Fazio, F., Calarese, D., Stevens, J., Razi, N., Stevens, D. J., Skehel, J. J., van Die, I., Burton, D. R., Wilson, I. A., Cummings, R., Bovin, N., Wong, C.-H., and Paulson, J. C. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 17033-17038.
5. Huflejt, M. E., Vuskovic, M., Vasiliu, D., Xu, H., Obukhova, P., Shilova, N., Tuzikov, A., Galanina, O., Arun, B., Lu, K., and Bovin, N. (2009) *Mol. Immunol.*, **46**, 3037-3049.
6. Yamamoto, T., Juneja, L. R., Hatta, H., and Kim, M. (1996) *Hen Eggs: Their Basic and Applied Science*, CRC Press, Boca Raton, USA.
7. Springer, G. F., Horton, R. E., and Forbes, M. (1959) *Ann. N. Y. Acad. Sci.*, **78**, 272-275.
8. Springer, G. F., and Horton, R. E. (1969) *J. Clin. Invest.*, **48**, 1280-1291.
9. Kaltgrad, E., Sen Gupta, S., Punna, S., Huang, C. Y., Chang, A., Wong, C. H., Finn, M. G., and Blixt, O. (2007) *ChemBioChem.*, **8**, 1455-1462.
10. Kuehn, A., Kovac, P., Saksena, R., Bannert, N., Klee, S. R., Ranisch, H., and Grunow, R. (2009) *Clin. Vaccine Immunol.*, **16**, 1728-1737.
11. Schwarzkopf, C., Staak, C., Behn, I., and Erhard, M. (2000) in *Chicken Egg Yolk Antibodies*, *Production and Application: IgY-Technology* (Schade, R., Behn, I., Erhard, M., Hlinak, A., and Staak, C., eds.) Springer, pp. 25-64.
12. Staak, C., Schwarzkopf, C., Behn, I., Hommel, U., Hlinak, A., Schade, R., and Erhard, M. (2000) in *Chicken Egg Yolk Antibodies*, *Production and Application: IgY-Technology* (Schade, R., Behn, I., Erhard, M., Hlinak, A., and Staak, C., eds.) Springer, pp. 65-107.
13. Consortium for Functional Glycomics (www.functionalglycomics.org).
14. Bacterial Carbohydrates Structure Database (http://www.glyco.ac.ru/bcsdb3/).
15. Baumgarth, N., Tung, J. M., and Herzenberg, L. A. (2005) *Springer Semin. Immunopathol.*, **26**, 347-362.