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Bo Diddley

Nel mezzo del cammin di nostra vita
mi ritrovai per una selva oscura
che la dirritta via era smarrita

Dante, Divina Commedia, Inferno

SELF, NON-SELF AND THE IMMUNE SYSTEM

ZELF, NIET-ZELF EN HET IMMUUNSYSTEEM

PROEFSCHRIFT

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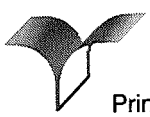

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PREAMBLE

A researcher is like a person in the library of Babel skimming the book of sand for the script of the godhead. In this search for knowledge a study of the immune system offers a challenging opportunity, since this group of cells reflects a behaviour which has many similarities with that of the people studying it. Although the repertoire of immunologists is much smaller than that of the lymphocytes in each one of them, the diversity of its publications yet puts the writer of a thesis on the behaviour of the immune system in the situation described in the first line. Lending an axiom from Glenn Gould, my introducer to Jorge Luis Borges - the writer of the stories mentioned in line one -, the only reason to write a thesis is to do it differently. With this in mind I have not chosen to write an encyclopedia with my own contributions in the center of the immunological universe, but to combine some of the publications I wrote during the last four years, to provide some theoretical background on the problems they address and to focus on the ideas they reflect.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

An adult mouse has a population of about 10^9 lymphocytes. These cells have clonally distributed cell surface receptors, which are generated by somatic rearrangements of DNA segments. The large number of these segments and the permutational nature of the recombinatory processes result in an immense collection of different receptors, the so-called "specificity repertoire". Based on their site of origin, their specificity repertoire and their function, two major lymphocyte subsets can be assigned: the B cells and the T cells. The lymphocyte surface receptors display ligand binding properties, which are a reflection of their own diversity and which allow the collections of clonal receptors to interact with anything that can be constructed at a molecular level. The members of this universe of molecular structures are called antigens, designating the clonal collections as antigen receptors. After a lymphocyte has encountered an antigen it can be induced to a variety of subset-specific responses. Due to the rather coincidental encounter of the natural sciences with some of these responses the family of lymphocytes has become known as the immune system. Since the start of Immunology the immune system has therefore been considered as a host-defense machine. As a consequence of this paradigm the ability to distinguish endogenous self-structures from molecules of exogenous origin has represented the central dogma of Immunology.

Based on the notion that it was improbable for a cellular organization like the immune system to have evolved from a process of selection by antigen-carrying intruders, Jerne indicated the importance of self-recognition for the evolutionary shaping and function of the immune system (1).

Coming from a microenvironment which was rich in Jernean stimuli, this thesis contains a few chapters which deal with reactions of the immune system to self- and non-self antigens.

Reference

1. Jerne, N.K. 1974. Ann. J. Immunol. (Inst. Pasteur) 125C: 373.

1.1 Development of B cells

B lymphocytes are produced in hematopoietic tissues from pluripotent hematopoietic stem cells (1). Fetal murine B lymphopoiesis is detected earliest in the placenta (2) and becomes evident in the fetal liver after 13 days of gestation (3,4). After birth, the bone marrow becomes the major site of B cell development (5,6). After development to a mature stage, B lymphocytes can differentiate into cells secreting immunoglobulins (Ig) of a unique antigen-binding specificity. Ig-molecules are made of two heavy and two light polypeptide chains, consisting of a constant and a variable part. The variable regions are responsible for the antigen-binding properties of the Ig-molecule, the constant parts determine the Ig-isotype. In the mouse, eight different isotypes (IgM, IgD, IgG1, IgG2a, IgG2b, IgG3, IgE, IgA) have been identified (7,8). Three gene families encode for mouse Ig-chains: κ light chain genes (9) located on chromosome 6, λ light chain genes (10) on chromosome 16 and heavy-chain genes (11) on chromosome 12. During the various stages of B cell development a series of events occur (12) that mediate a somatic rearrangement of the Ig gene clusters (13). During this process gene segments encoding constant regions are joined to a combination of the various gene segments encoding the variable region of the corresponding Ig chain. An Ig-molecule is assembled by a conjugation of two pairs of light and heavy chains. The multitude of genes encoding Ig variable regions, the permutational nature of the somatic rearrangements and the putative random association of heavy and light chains are responsible for a highly diverse repertoire of different immunoglobulins (14).

In the adult mouse 3 to 5 x 10^7 B cells are newly formed each day in the bone marrow (6). The great majority migrates to the peripheral lymphoid organs like spleen and lymph nodes. This production is so high that within 2-4 days enough new B lymphocytes are produced to replenish the whole peripheral compartment (15). A small proportion of the mature B cells differentiate to clones of Ig-secreting cells. A total of 1 to 2 x 10^6 Ig-secreting cells can be found in spleen, bone marrow and lymph nodes (16) and about 2 x 10^7 secreting mostly IgA in the small intestine (17).

References

1. Müller-Sieburg, C.E. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 331.
2. Melchers, F. 1979. Nature 277: 219.
3. Raff, M.C., Megson, M., Owen, J.J.T. and Cooper, M.D. 1976. Nature 259: 224.
4. Phillips, R.A. and Melchers, F. 1976. J. Immunol. 117: 1099.
5. Osmond, D.G. 1980. In: Immunoglobulin genes and B cell differentiation, eds. J.R. Battisto and J.L. Knight, Elsevier-North Holland, New York, p. 135.
6. Osmond, D.G. 1986. Immunol. Rev. 93: 103.

7. Edelman, G.M. 1973. Science 180: 830.
8. Hasemann, C.A. and Capra, J.D. 1989. In: Fundamental immunology, ed. W.E. Paul, Raven Press, New York, p. 209.
9. Swan, D., D'Eustachio, P., Leinwand, L., Seidman, J., Keithley, D. and Ruddle, F.H. 1979. Proc. Natl. Acad. Sci. USA 76: 2735.
10. D'Eustachio, P., Bothwell, A.L.M., Takaro, T.K., Baltimore, D. and Ruddle, F.H. 1981. J. Exp. Med. 153: 793.
11. D'Eustachio, P., Pravtcheva, D., Marcu, K. and Ruddle, F.H. 1980. J. Exp. Med. 151: 1545.
12. Baltimore, D., Oettinger, M. and Schatz, D. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 385.
13. Phillips, R.A. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 305.
14. Tonegawa, S. 1983. Nature 302: 575.
15. Freitas, A., Pereira, P., Huetz, F., Thomas-Vaslin, V., Pena-Rossi, C., Andrade, L., Sundblad, A., Forni, L. and Coutinho, A. 1989. Contrib. Microbiol. Immunol. 11: 1.
16. Benner, R., van Oudenaren, A., Björklund, M., Ivars, F. and Holmberg, D. 1982. Immunol. Today 3: 243.
17. Van der Heijden, P.J., Stok, W. and Bianchi, A.T.J. 1987. Immunology 62: 551.

1.2 Major histocompatibility complex

The major histocompatibility complex (MHC) is a multigene family whose members encode cell surface glycoproteins involved in the recognition of and in the immune response to antigens. The murine MHC, called the H-2 locus, is located on chromosome 17 (1) and contains various classes of genes (2). Two groups of products of the H-2 locus, the MHC class I and class II molecules, will be described here in some detail.

The class I molecules, H-2D, H-2K and H-2L, are highly polymorphic (3), consist of an H-2 encoded glycosylated polypeptide chain non-covalently associated with β2-microglobulin, a small polypeptide encoded on chromosome 2, and are expressed on the surface of both lymphoid and non-lymphoid cells in varying densities. X-ray crystallographic structure determination of the human HLA-A2 molecule has revealed that the α3 and β2-microglobulin domains are associated and both have the structure of an immunoglobulin-like domain, whereas the α1 and α2 domains form a symmetrical structural unit consisting of a platform composed of a β-pleated sheet topped by two α-helices with a long groove between them (4). This groove contains most of the polymorphic residues and provides a binding site for antigens (5). The class II molecules consist of two non-covalently associated peptides, an α heavy chain and a β light chain (6) encoded by genes of the H-2 I-A and I-E regions (7,8). A prediction of the three-dimensional structure of class II molecules indicated a high similarity with the class I molecules, including the presence of an antigen-binding

groove (9).

In contrast to the class I molecules, class II molecules are constitutively expressed on a limited number of cell types like B cells (10,11), macrophages (12), interdigitating reticular cells (13) and Langerhans cells (14) and can be induced on several other celltypes like fibroblasts (15), endothelial cells (16), astrocytes (17) and thymic epithelial cells (18).

References

1. Klein, J. 1975. Biology of the mouse histocompatibility-2 complex, Springer-Verlag, New York.
2. Hood, L., Steinmetz, M. and Malissen, B. 1983. Ann. Rev. Immunol. 1: 529.
3. Klein J., Figueroa, F. and David, C.S. 1983. Immunogenetics 17: 553.
4. Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. 1987. Nature 329: 506.
5. Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. 1987. Nature 329: 512.
6. Klein, J., Figueroa, F. and Nagy, Z.A. 1983. Ann. Rev. Immunol. 1: 119.
7. Klein, J., Juretic, A., Baxevanis, C.N. and Nagy, Z.A. 1981. Nature 291: 455.
8. Jones, P.P., Murphy, D.B. and McDevitt, H.O. 1978. J. Exp. Med. 148: 925.
9. Brown, J.H., Jardetzky, T., Saper, M.A., Samraoui, B., Bjorkman, P.J. and Wiley, D.C. 1988. Nature 332: 845.
10. Glimcher, L.H., Hamano, T., Asofsky, R., Heber-Katz, E., Hedrick, S., Schwartz, R.W. and Paul, W.E. 1982. Nature 298: 283.
11. Ashwell, J.D., DeFranco, A.L., Paul, W.E. and Schwartz, R.H. 1984. J. Exp. Med. 159: 881.
12. Schwartz, R.H., Yano, A. and Paul, W.E. 1978. Immunol. Rev. 40: 153.
13. Sunshine, G.H., Gold, D.P., Wortis, H.H., Marrack, P. and Kappler, J.W. 1983. J. Exp. Med. 158: 1745.
14. Stingl, G., Tamaki, K. and Katz, S.I. 1980. Immunol. Rev. 53: 149.
15. Pober, J.S., Collins, T., Gimbrone, M.A., Jr., Cotran, R.S., Gitlin, J.D., Fiers, W., Clayberger, C., Krensky, A.M., Burakoff, S.J. and Reiss, C.S. 1983. Nature 305: 726.
16. Groenewegen, G., Buurman, W.A. and van der Linden, C.J. 1985. Nature 316: 361.
17. Massa, P.T., Dörries, R. and ter Meulen, V. 1986. Nature 320: 543.
18. Berrih, S., Arenzana-Seisdedos, F., Cohen, S., Devos, R., Charron, D., Virelizier, J.L. 1985. J. Immunol. 135: 1165.

1.3 Processing and presentation of antigens

Structure determinations of MHC class I and class II have revealed the presence of a long groove in these molecules, which can bind antigen (1,2) and *in vitro* studies employing purified MHC molecules have shown their association with antigenic peptides (3-5). Before antigens can associate with MHC molecules they must be modified by a mechanism called antigen processing, involving their proteolytic degradation into small peptide fragments (6). After degradation these fragments have

an important role in the association of the heavy and light chains of the MHC molecules and their consequent transport to, and stabilisation on the cell surface (7,8). It is therefore supposed that all MHC molecules which are present on a cell surface, carry an antigenic peptide (9). There have been indications that MHC class I molecules associate with peptides from endogenous antigens, whereas class II molecules form complexes with peptides from exogenous antigens taken up by the cell (10-12), but this distinction is not absolute (13,14).

Analysis of the binding of panels of peptides to mouse I-A^d has shown the broad specificity of these molecules (15), which allows different peptides to compete for MHC-binding (16). This will lead to the presentation of a diverse set of peptides on each cell, including self-peptides (17).

References

1. Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. 1987. Nature 329: 506.
2. Brown, J.H., Jardetzky, T., Saper, M.A., Samraoui, B., Bjorkman, P.J. and Wiley, D.C. 1988. Nature 332: 845.
3. Babbit, B.P., Allen, P.M., Matsueda, G., Haber, E. and Unanue, E.R. 1985. Nature 317: 359.
4. Buus, S., Sette, A., Colon, S.M., Jenis, D.M. and Grey, H.M. 1986. Cell 47: 1071.
5. Sadegh-Nasseri, S. and McConnell, H.M. 1989. Nature 337: 274.
6. Unanue, E.R. 1984. Ann. Rev. Immunol. 2: 395.
7. Townsend, A., Öhlén, C., Bastin, J., Ljunggren, H.-G., Foster, L. and Kärre, K. 1989. Nature 340: 443.
8. Ljunggren, H.-G., Stam, N.J., Öhlén, C., Neefjes, J.J., Höglund, P., Heemels, M.-T., Bastin, J., Schumacher, T.N.M., Townsend, A., Kärre, K. and Ploegh, H.L. 1990. Nature 346: 476.
9. Germain, R.N. 1990. Nature 344: 19.
10. Morrison, L.A., Lukacher, A.E., Braciale, V.L., Fan, D.P. and Braciale, T.J. 1986. J. Exp. Med. 163: 903.
11. Germain, R.N. 1986. Nature 322: 687.
12. Sweetser, M.T., Morrison, L.A., Braciale, V.L. and Braciale, T.J. 1989. Nature 342: 180.
13. Sekaly, R.P., Jacobson, S., Richert, J.R., Tonnelle, C., McFarland, H.F. and Long, E.O. 1988. Proc. Natl. Acad. Sci. USA 85: 1209.
14. Nuchtern, J.G., Biddison, W.E., Klausner, R.D. 1990. Nature 343: 74.
15. Sette, A., Buus, S., Colon, S., Smith, J.A., Miles, C. and Grey, H.M. 1987. Nature 328: 395.
16. Adorini, L. and Nagy, Z.A. 1990. Immunol. Today 11: 21.
17. Lorenz, R.G. and Allen, P.M. 1988. Proc. Natl. Acad. Sci. USA 85: 5220.

1.4 Development of T cells

The majority of T lymphocytes is produced in the thymus from hematopoietic precursor cells (1). During murine fetal development the thymus is seeded by precursor cells from the fetal liver in two waves, one at 10 days (2), the other at 18

days (3) of gestation. After birth the bone marrow becomes the source of thymic precursor cells (4). These are called pro-T lymphocytes (5) or prothymocytes (6). The thymus, an epithelial organ derived from the third and fourth pharyngeal pouches, is indispensable for the generation of functional T cells (7-9). It contains endo- and ectodermal tissues (epithelium) as well as bone marrow derived cells (macrophages and dendritic cells). Anatomically, the thymus can be divided in a cortex, where most of the thymocytes and most of the proliferative activity can be found, and a medulla which contains lymphocytes with a mature phenotype (10). On the basis of the expression of the CD4 and CD8 surface molecules, thymocytes can be divided into four subsets. The double negative population, about 1 to 2% of all thymocytes, is concentrated under the capsule in the outer cortex, reflects the earliest stage of thymocyte development and has the ability to repopulate the thymus (11,12). The double positive population, about 80 to 85% of all thymocytes, represents an intermediate stage of thymocyte development (13,14). The remaining 10 to 15% of thymocytes compose a mixture of single-positive $CD4^+$ and $CD8^+$ cells, which are immunocompetent and largely found in the medulla, from where they are exported to peripheral lymphoid tissues. Besides CD4 and CD8, CD3 and the T cell receptor (TcR) are expressed during thymocyte development (15-17). Double-positive thymocytes express low-to-intermediate levels of CD3 and TcR, whereas most single-positive cells express these molecules at a high level. In the mouse two types of TcR, the TcR-$\alpha\beta$ and the TcR-$\gamma\delta$, are expressed, which are composed of two chains, consisting of both constant and variable parts. Four gene families encode for the murine TcR-chains: α located on chromosome 14 (18), β located on chromosome 6 (19), γ located on chromosome 13 (20), and δ located within the α-locus on chromosome 14 (21). T cells carrying the TcR-$\gamma\delta$ represent a separate lineage (22), predominate in epithelia (23), and have been demonstrated to react to bacterial surface antigens (24,25). Their function is, however, still unclear and it is beyond the aim of this introduction to deal with them in more detail.

During their development, thymocytes rearrange the genes encoding the TcR-chains (26) and express the resulting receptor on their cell surface (15-17). The reactivity of the TcR-$\alpha\beta$ repertoire is carefully screened by two mechanisms called positive and negative selection. These selections result in a population of mature thymocytes, which has been educated to respond to peptides presented in the context of self-MHC molecules and from which cells carrying receptors with a too high extent of autoreactivity have been deleted (27).

Negative selection, also known as clonal deletion, prevents double positive thymocytes which display self-reactivity to differentiate to mature single positive T cells (28). Self-antigens for which clonal deletion has been demonstrated include MHC I-E molecules (29,30), the minor lymphocyte stimulating antigens (MLS) (31,32) and the

male-specific HY antigen (33). Although HY is the only "conventional" antigen for which clonal deletion has been demonstrated, negative selection is generally considered of major importance for a state of immunological self-tolerance (34).

Positive selection allows double positive cells carrying T cell receptors which display appropriate reactivity to self-MHC-peptide complexes to differentiate into a more mature stage (35,36). During positive selection interactions between MHC class I and CD8 (37-39) and between MHC class II and CD4 (40-42) play a crucial role in the differentiation to mature thymocytes single-positive for CD8 and CD4, respectively. There is some evidence that positive selection precedes clonal deletion (43) and that the recognition of presented self-peptides may be involved in this process (44).

The thymus of an adult mouse releases about 1 x 10^6 cells each day (45). Experiments employing the antimitotic drug hydroxyurea have indicated that at least half of the peripheral T cells is constituted by lymphocytes which have divided 24-48 hours previously (46). These findings indicate the importance of peripheral renewal for the maintenance of the T cell pool, a process in which an important role for self-peptides has recently been demonstrated (47).

References

1. Owen, J.J.T., Jenkinson, E.J., Kingston, R., Wiliams, G.T. and Smith, C.A. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 215.
2. Owen, J.J.T., Wright, D.E., Habu, S., Raff, M.C. and Cooper, M.D. 1977. J. Immunol. 118: 2067.
3. Jotereau, F., Hueze, F., Salomon-Vie, V. and Gascan, H. 1987. J. Immunol. 138: 1026.
4. Lepault, F., Coffman, R.L. and Weissman, I.L. 1983. J. Immunol. 131: 64.
5. Palacios, R., Kiefer, M., Brockhaus, M., Karjalainen, K., Dembić, Z., Kisielow, P. and von Boehmer, H. 1987. J. Exp. Med. 166: 12.
6. Mulder, A., Visser, J.W., Zoetelief, J. and van Bekkum, D.W. 1988. Thymus 11: 29.
7. Miller, J.F.A.P. and Osoba, D. 1967. Physiol. Rev. 47: 437.
8. Hodes, R.J., Sharrow, S.O. and Solomon, A. 1989. Science 246: 1041.
9. Fry, A.M., Jones, L.A., Kruisbeek, A.M. and Matis, L.A. 1989. Science 246: 1044.
10. Sprent, J. 1989. In: Fundamental Immunology, ed. W.E. Paul, Raven Press, New York, p. 69.
11. Ezine, S., Weissman, I.L. and Rowse, R.V. 1984. Nature 309: 629.
12. Fowlkes, B.J., Edison, L., Mathieson, B.J. and Chused, T.M. 1985. J. Exp. Med. 162: 802.
13. Kyewski, B.A., Momburg, F. and Schirrmacher. 1987. Eur. J. Immunol. 17: 961.
14. MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M. and Hengartner, H. 1988. Nature 332: 40.
15. MacDonald, H.R., Howe, R.C., Pedrazzini, T., Lees, R.K., Budd, R.C., Schneider, R., Liao, N.S., Zinkernagel, R.M., Louis, J.A., Raulet, D.H., Hengartner, H. and Miescher, G. 1988. Immunol. Rev. 104: 157.
16. Roehm, N., Herron, L., Cambier, J., Diguisto, D., Haskins, K., Kappler, J. and Marrack, P. 1984. Cell 38: 577.
17. von Boehmer, H., Karjalainen, K., Pelkonen, J., Borgulya, P. and Rammensee, H.-G. 1988. Immunol. Rev. 101: 21.
18. Epstein, R., Roehm, N., Marrack, P., Kappler, J., Davis, M., Hedrick, S. and Cohn, M. 1985. J.

Exp. Med. 161: 1219.
19. Caccia, N., Kronenberger, M., Saxe, D., Haars, R., Bruns, G.A.P., Goverman, J., Malissen, M., Willard, H., Yoshikai, Y., Simon, M., Hood, L. and Mak, T.W. 1984. Cell 37: 1091.
20. Kranz, D.M., Saito, H., Disteche, C.M., Swisshelm, K., Pravtcheva, D., Ruddle, F.H., Eisen, H.N. and Tonegawa, S. 1985. Science 227: 941.
21. Chien, Y., Iwashima, M., Kaplan, K.B., Elliott, J.F. and Davis, M.M. 1987. Nature 327: 677.
22. Winoto, A. and Baltimore, D. 1989. Nature 338: 430.
23. Tonegawa, S., Berns, A., Bonneville, M., Farr, A.G., Ishida, I., Ito, K., Itohara, S., Janeway, C.A., Jr., Kanagawa, O., Katsuiki, M., Kubo, R., Lafaille, J.J., Mombaerts, P., Murphy, D.B., Nakansihi, N., Takagaki, Y., van Kaer, L. and Verbeek, S. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 243.
24. Janis, E.M., Kaufmann, S.H.E., Schwartz, R.H. and Pardoll, D.M. 1989. Science 244: 713.
25. Rust, C.J.J., Verreck, F., Vietor, H. and Koning, F. 1990. Nature 346: 572.
26. Kronenberg, M., Siu, G., Hood, L.E. and Shastri, N. 1986. Ann. Rev. Immunol. 4: 529.
27. von Boehmer, H., Teh, H.S. and Kisielow, 1989. Immunol. Today 10: 57.
28. Fowlkes, B.J., Schwartz, R.H. and Pardoll, D.M. 1988. Nature 334: 620.
29. Kappler, J.W., Roehm, N. and Marrack, P. 1987. Cell 49: 273.
30. Bill, J., Kanagawa, O., Woodland, D.L. and Palmer, E. 1989. J. Exp. Med. 169: 1405.
31. Pullen, A.M., Marrack, P. and Kappler, J.W. 1988. Nature 335: 796.
32. Abe, R. and Hodes, R.J. 1989. Ann. Rev. Immunol. 7: 683.
33. Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. 1988. Nature 333: 742.
34. Blackman, M., Kappler, J. and Marrack, P. 1990. Science 248: 1335.
35. Teh, H.S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Blüthmann, H. and von Boehmer, H. 1988. Nature 335: 229.
36. Sha, W.C., Nelson, C.A., Newberry, R.D., Kranz, D.M., Russell, J.H. and Loh, D.Y. 1988. Nature 336: 73.
37. Marusić-Galesić, S., Stephany, D.A., Longo, D.L. and Kruisbeek, A.M. 1988. Nature 333: 180.
38. Zúñiga-Pflücker, J.C., Jones, L.A., Longo, D.L. and Kruisbeek, A.M. 1990. J. Exp. Med. 171: 427.
39. Zijlstra, M., Bix, M., Simister, N., Loring, J.M., Raulet, D.H. and Jaenisch, R. 1990. Nature 344: 742.
40. Kruisbeek, A.M., Fultz, M.J., Sharrow, S.O., Singer, A. and Mond, J.J. 1983. J. Exp. Med. 157: 1932.
41. Kruisbeek, A.M., Mond, J.J., Fowlkes, B.J., Carmen, J.A., Bridges, S.A. and Longo, D.L. 1985. J. Exp. Med. 161: 1029.
42. Zúñiga-Pflücker, J.C., McCarthy, S.A., Weston, M., Longo, D.L., Singer, A. and Kruisbeek, A.M. 1989. J. Exp. Med. 169: 2085.
43. Ohashi, P.S., Pircher, H., Bürki, K., Zinkernagel, R.M. and Hengartner, H. 1990. Nature 346: 861.
44. Nikolić-Zugić, J. and Bevan, M.J. 1990. Nature 344: 65.
45. Shortman, K. and Scollay, R. 1985. In: Recognition and Regulation in Cell-Mediated Immunity, eds. J.D. Watson and J. Marbrook, Marcel Dekker, New York, p. 31.
46. Rocha, B., Freitas, A.A. and Coutinho, A.A. 1983. J. Immunol. 131: 2158.
47. Rocha, B. and von Boehmer, H. Personal communication.

1.5 Cellular immune responses

Thymic development results in two major populations of T cells: CD8-positive cells reacting with specific peptides presented in the context of MHC class I molecules and CD4-positive cells showing MHC class II restriction (1).

Two major functions of CD8-positive cells have been demonstrated: cytotoxicity mediated by cytotoxic T cells (2) and suppression mediated by suppressor T cells (3). Cytotoxic T cells play a crucial part in the cellular immune response and have the ability to lyse cells presenting peptides of viral origin (4).

"Suppressor T cells" have been defined as cells that can specifically suppress immune responses by antigen-specific suppressor-factors. The difficulty in cloning these cells, the inability to clone their suppressor-factors, the difficulty in finding their MHC restriction and in excluding the role of external influences on the suppression found in a variety of experimental systems, has made the suppressor T cell to the most debated and least accepted cell of the immune system. The demonstration of the secretion of a number of cytokines - non-antigen-specific T cell factors - by clones of cytotoxic cells (5), may open new ways to understand the suppressive effects mediated by CD8-positive T cells.

CD4-positive T cells can be induced to secrete a variety of cytokines, which can enhance a variety of immune reactions, and are therefore known as helper T cells. Although the precise details are still unclear, mature CD4-positive T cells are thought to be induced to the differentiation to the T_{H0} stage after antigen-recognition (5). From this stage the cells can be induced to differentiate to T_{H1}- and T_{H2}-cells, which are able to secrete distinct patterns of cytokines. These patterns, which have been established by the analysis of panels of helper T cell clones (6), seem to reflect the functions of the two subsets: T_{H1}-cells being responsible for delayed type hypersensitivity reactions, producing cytokines like interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor; T_{H2}-cells playing a major role in the clonal growth and differentiation of B cells, producing the interleukins 4, 5 and 10.

References

1. Swain, S. 1983. Immunol. Rev. 74: 129.
2. Zinkernagel, R.M. and Doherty, P.C. 1979. Adv. Immunol. 27: 51.
3. Dorf, M.E. and Benacerraf, B. 1984. Ann. Rev. Immunol. 2: 127.
4. Doherty, P.C., Tabi, Z., Cleary, A., Allan, W. and Carding, S. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 897.
5. Mosmann, T.R., Street, N.E., Fiorentino, D.F., Bond, M.W., Fong, T.A.T., Schumacher, J., Leverah, J.A., Trounstine, M., Vieira, P. and Moore, K.W. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/Heidelberg, p. 611.
6. Mosmann, T.R. and Coffman, R.L. 1987. Immunol. Today 8: 223.

1.6 Humoral immune responses

Besides the cellular immune response mediated by cytotoxic T cells, the humoral immune response, mediated by specific antibodies, is the second antigen-specific mechanism by which the immune system can protect the host against infectious agents. Responses of B lymphocytes to most protein antigens are dependent upon the helper function of T lymphocytes (1). In order to provide this support, CD4-positive T cells must be activated by the presentation of specific peptides in the context of an MHC class II molecule (2). The two functionally distinct candidates for antigen presentation are the antigen-specific B cell, which is ultimately stimulated to antibody secretion, and distinct populations of antigen-non-specific cells like macrophages and dendritic cells (3). The relative importance of these two classes of cells is highly dependent upon the concentration of antigen available for presentation, since it has been shown that antigen-specific B cells can effectively present antigen at a thousand-fold lower concentration than macrophages (4).

After activation the helper T cells can be induced to secrete a variety of cytokines, which can induce antigen-specific B cells to differentiation and proliferation (5). As a consequence of this activation, B cells can change the isotype of the immunoglobulin which they synthesize from IgM to one of the other isotypes, a process known as isotype switching (6). It has been demonstrated that certain cytokines can mediate the switching to a specific isotype: interleukin-4 inducing IgG1 and IgE, interleukin-5 inducing IgA and IFN-γ inducing IgG2a (7). Cytokines can also inhibit the production of particular isotypes, e.g. IgE by IFN-γ. During the humoral immune response two other processes occur which are important for host-defense: somatic mutation of the variable regions of the immunoglobulin molecules, causing an increased affinity of the antibody binding site for the antigen (8), and the generation of an antigen-specific memory, causing an accelerated and increased response after the second encounter with the same antigen (9).

References

1. Hodes, R.J. 1989. In: Fundamental Immunology, ed. W.E. Paul, Raven Press, New York, p. 587.
2. Schwartz, R.H. 1985. Ann. Rev. Immunol. 3: 237.
3. Hodes, R.J. 1989. Sem. Immunol. 1: 23.
4. Abbas, A.K., Haber, S. and Rock, K.L. 1985. J. Immunol. 135: 1661.
5. Paul, W.E. 1989. In: Progress in Immunology VII, ed. F. Melchers, Springer-Verlag, Berlin/ Heidelberg, p. 553.
6. Radbruch, A., Burger, C., Klein, S. and Müller, W. 1986. Immunol. Rev. 89: 69.
7. Coffman, R.L., Seymour, B.W.P., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Schrader, B., Cherwinsky, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W. and Mosmann, T.R. 1988. Immunol. Rev. 102: 5.

8. Wysocki, L., Manser, T. and Gefter, M.L. 1986. Proc. Natl. Acad. Sci. USA 83: 1847.
9. Cumano, A. and Rajewsky, K. 1986. EMBO J. 5: 2459.

1.7 Self, non-self and the immune system

About a century ago the important role of the immune system in host defense against infectious agents was established (1). Already in the very early days of Immunology it was hypothesized that a host defense system should be able to make a fair distinction between self and non-self antigens in order to avoid a "horror autotoxicus" (2). Ninety years of exponentially expanded immunological research later, "the mechanism of self versus non-self recognition in the immune system ranks at or near the top of all the mysteries of modern science" (3). In order to prevent T cell autoreactivity, three mechanisms have been proposed (4): clonal deletion (5), causing a removal of autoreactive cells during thymocyte development, clonal anergy (6), causing a functional inactivation of mature T cells displaying autoreactivity, and suppression (7) mediated by self-specific suppressor cells. Clonal deletion is the only one of these which is currently supported by results from various *in vivo* studies and which has been demonstrated for different antigens (8-10). The demonstration of self-reactive clones among mature lymphocytes in several experimental *in vitro* systems (11-13) has, however, indicated that T cells with autoreactive properties may be present in normal individuals. Mechanisms preventing the production of autoreactive antibodies have been documented to a lesser extent than their T cell counterparts. An *in vivo* model of double-transgenic mice, which at the one hand produce the *neo self-antigen* hen egg lysozyme and at the other hand express a high affinity antibody to this antigen on the B cell surface, has shown a functional silencing of these B cells (14). This process of silencing, however, occurred only at high antigen concentrations (15). The broad range of low affinity interactions with self-antigens displayed by the natural antibodies of unmanipulated mice (16) yet indicated that self-reactive B cells are present under normal physiological conditions.

Based on the idea that the specificity repertoire of all Ig is so broad that it must also include reactivity against antigenic sites on the variable regions of the Ig themselves, the so-called idiotypes, Jerne has formulated the idiotypic network theory (17). The results of a variety of immunization experiments have indicated the generation of anti-idiotypic antibodies during immune responses (18-22) and idiotypic-connectivity has been demonstrated within the B cell pool of unmanipulated mice (23,24). Idiotypic interactions between B and T cells have been demonstrated (25) but the precise mechanisms and the relative importance of these interactions within the normal immune system is still unclear (26).

The concept of an idiotypic network has been extensively debated (26) and adjustments like connective networks have been proposed (27), but the central idea of the network theory: the active recognition of self-structures by the immune system (28), has not lost much of its value. A direct consequence of this network theory has been the proposition, that host defense is one among the tasks of the immune system, which could function as a kind of self-referential system (29).

References

1. Grabar, P. 1984. In: Basic and Clinical Immunology, eds. D.P. Stites, J.D. Stobo, H.H. Fudenberg and J.V. Wells, Lange, Los Altos, p. 1.
2. Ehrlich, P. and Morgenroth, J. 1957. Reprinted in: The collected papers of Paul Ehrlich, Vol. 2, eds. F. Himmelweit, M. Marquardt and H.D. Dale, Pergamon, London, Vol. 2, p. 205.
3. Koshland, D.E., Jr. 1990. Science 248: 1273.
4. Blackman, M., Kappler, J. and Marrack, P. 1990. Science 248: 1335.
5. Kappler, J.W., Roehm, M. and Marrack, P. 1987. Cell 49: 273.
6. Jenkins, M.K., Pardoll, D.M., Mitzuguchi, J., Chused, T.M. and Schwartz, R.H. 1987. Proc. Natl. Acad. Sci. USA 84: 5409.
7. Miller, R.D. and Calkins, C.E. 1988. J. Immunol. 140: 3779.
8. Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. 1988. Nature 333: 742.
9. Fowlkes, B.J., Schwartz, R.H., Pardoll, D.M. 1988. Nature 334: 620.
10. MacDonald, H.R., Hengartner, H. and Pedrazzini, T. 1988. Nature 335: 174.
11. Smith J.B. and Pasternak, R.D. 1978. J. Immunol. 121: 1889.
12. Battisto, J.R. and Ponzio, N.M. 1981. Prog. Allergy 28: 169.
13. Glimcher, L.H. and Shevach, E.M. 1982. J. Exp. Med. 156: 640.
14. Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., Trent, R.J. and Basten, A. 1988. Nature 334: 676.
15. Goodnow, C.C., Adelstein, S. and Basten, A. 1990. Science 248: 1373.
16. Dighiero, G., Lymberi, P., Mazié, J.-C., Rouyre, S., Butler-Browne, G.S., Whalen, R.G. and Avrameas, S. 1983. J. Immunol. 131: 2267.
17. Jerne, N.K. 1974. Ann. Immunol. (Inst. Pasteur) 125C: 373.
18. Cosenza, H. 1976. Eur. J. Immunol. 6: 114.
19. Urbain, J., Wikler, M., Franssen, J.D. and Collignon, C. 1977. Proc. Natl. Acad. Sci. USA 74:5126.
20. Jerne, N.K., Roland, J. and Cazenave, P.-A. 1982. EMBO J. 1: 243.
21. Couraud, P.-O., Lü, B.-Z. and Strosberg, A.D. 1983. J. Exp. Med. 157: 1369.
22. Schiff, C., Milili, M., Hue, I., Rudikoff, S. and Fougereau, M. 1986. J. Exp. Med. 163: 573.
23. Holmberg, D., Forsgren, S., Ivars, F. and Coutinho, A. 1984. Eur. J. Immunol. 14: 435.
24. Kearney, J.F., Vakil, M. and Nuhobson, N. 1987. In: Evolution and Vertebrate Immunity: the Antigen Receptor and MHC Gene Families, eds. G. Kelsoe and D. Schulze, Texas University Press, Austin, p. 175.
25. Sim, G.-K., MacNeil, I.A. and Augustin, A.A. 1986. Immunol. Rev. 90: 49.
26. Langman, R.E. and Cohn, M. 1986. Immunol. Today 7: 100.
27. Coutinho, A. 1989. Immunol. Rev. 110: 63.
28. Jerne, N.K. 1984. Immunol. Rev. 79: 5.
29. Coutinho, A., Forni, L., Holmberg, D., Ivars, F. and Vaz, N. 1984. Immunol. Rev. 79: 151.

CHAPTER 2

INTRODUCTION TO THE EXPERIMENTAL WORK

An important aspect of the idiotypic network theory (1) was the idea of an eigen-behaviour within the immune system, a dynamic steady state in the absence of antigens that do not belong to the system. This concept was an incentive to study the immune system of not intentionally immunized individuals. These studies have indicated the presence of "background" immunoglobulin secreting cells in all lymphoid organs of unmanipulated mice (2,3) as well as activated T cells (4). Studies on the kinetics of these cells indicated the high renewal rate of both background immunoglobulin secreting cells (IgSC) (5,6) and T lymphocytes (7). The influence of exogenous antigenic stimulation by dietary antigens and by bacterial and viral products on the observed eigen-behaviour of the immune system could, however, not be excluded. In order to circumvent this influence, the immune system of germfree (GF) mice fed a chemically defined (CD) ultrafiltered diet of low molecular weight nutrients has been studied. These mice live under sterile conditions, are free from viruses and are fed a diet which can be considered free from substances with antigenic properties (8). Studies of the B cell compartment of these so-called GF-CD mice have been done by my predecessors Herbert Hooijkaas and Nico Bos (9-16). These studies indicated that GF-CD mice possess normal numbers of cells secreting IgM in all lymphoid tissues but severely reduced numbers of cells secreting other isotypes, which show a good correlation with the presence of these Ig in the serum. Isotype switching could, however, be induced by *in vitro* culture of GF-CD B cells in the presence of the mitogenic substance lipopolysaccharide. Analysis of the specificity of the B cells of GF-CD mice and their usage of Ig V_H genes has shown their similarity with B cells from conventional neonatal mice.

Based on a report on the presence of T cells in GF-CD mice (17), Chapter 4 describes a study of the development of T cells in these mice. The main questions addressed in this chapter are:

1. Can T cells develop in a physiological environment which has endogenous antigens as the only source of peptides involved in positive and negative selection of thymocytes?
2. Can quantitative or qualitative differences be detected between T cell

development in GF-CD and conventional mice?
3. Can quantitative or qualitative differences be detected between peripheral selection of T cell repertoires in GF-CD and in conventional mice?
4. Can T cells of GF-CD mice display functional properties?

Based on observation of severely reduced numbers of IgSC of non-IgM isotypes (10,13), whose isotype switching is induced by various lymphokines of helper T cell origin (18), Chapter 5 addresses the following questions:

1. Can GF-CD T cells respond to allogeneic stimuli? (These responses were chosen since they are induced by the recognition of MHC molecules and associated peptides via the T cell receptor of the responding cells and thus allow a direct conclusion of the functional capacity of GF-CD T cells).
2. Can GF-CD T cells be induced to produce lymphokines?
3. Can the factors produced by stimulated GF-CD T cells induce isotype switching in B cells of the same origin?
4. Can activated T cells be detected in the peripheral lymphoid tissues of GF-CD mice?

The MHC class II molecules are of critical importance for the intercellular communication between helper T cells and cells with an antigen-presenting function (19). Studies on the *in vivo* treatment of mice with specific antibodies against their MHC class II molecules have shown the disappearance of these molecules from the surface of antigen-presenting cells with a concomitant loss of antigen-presenting function (20,21). The extent of the effects of *in vivo* treatment with anti-MHC class II may be illustrated by the blockade found in the development of CD4-positive cells after an administration to neonatal mice (22,23).

Conventional mice have background IgSC of various isotypes in their lymphoid organs (2,3). In order to study the mechanisms inducing B cells to this background secretion, Chapter 6 describes a study of the effects of *in vivo* treatment with anti-MHC class II antibodies on this process.

The following questions are addressed:

1. Are intercellular interactions via MHC class II molecules involved in the induction of background immunoglobulin production?
2. Does treatment with anti-MHC class II antibodies affect the pool of peripheral T cells?
3. Which are the kinetics of the effects of the treatment and of the subsequent recovery of the immune system?

Chapter 3 contains the protocol of the technique used in the subsequent chapters for the detection of IgSC. Moreover, this chapter describes a technique resulting from earlier studies (24,25), which allows an improved detection of cells secreting specific antibodies and presents results from a "classical" immunization study.

References

1. Jerne, N.K. 1974. Ann. Immunol. (Inst. Pasteur) 125C: 373.
2. Benner, R., Rijnbeek, A.-M., Bernabé, R.R., Martinez-A., C. and Coutinho, A. 1981. Immunobiol. 158: 225.
3. Benner, R., van Oudenaren, A., Björklund, M., Ivars, F. and Holmberg, D. 1982. Immunol. Today 3: 243.
4. Pereira, P., Larsson, E.-L., Forni, L., Bandeira, A. and Coutinho, A. 1985. Proc. Natl. Acad. Sci. USA 82: 7691.
5. Ho, F., Lortan, J.E., MacLennan, I.C. and Khan, M. 1986. Eur. J. Immunol. 16: 1297.
6. Lévy, M., Vieira, P., Coutinho, A. and Freitas, A. 1987. Eur. J. Immunol. 17: 849.
7. Rocha, B., Freitas, A.A. and Coutinho, A.A. 1983. J. Immunol. 131: 2158.
8. Pleasants, J.R., Johnson, M. and Wostmann, B.S. 1986. J. Nutr. 116: 1949.
9. Hooijkaas, H., Benner, R., Pleasants, J.R. and Wostmann, B.S. 1984. Eur. J. Immunol. 14: 1127.
10. Hooijkaas, H., van der Linde-Preesman, A.A., Bitter, W.M., Benner, R., Pleasants, J.R. and Wostmann, B.S. 1985. J. Immunol. 134: 2223.
11. Bos, N.A., Benner, R., Wostmann, B.S. and Pleasants, J.R. 1986. J. Reprod. Immunol. 9: 237.
12. Bos, N.A., Meeuwsen, C.G., Hooijkaas, H., Benner, R., Wostmann, B.S. and Pleasants, J.R. 1987. Cell. Immunol. 105:235.
13. Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R. and Benner, R. 1988. Cell. Immunol. 112: 371.
14. Bos, N.A. and Meeuwsen, C.G. 1989. Eur. J. Immunol. 19:1811.
15. Bos, N.A., Meeuwsen, C.G., van Wijngaarden, P. and Benner, R. 1989. Eur. J. Immunol. 19: 1817.
16. Bos, N.A., Kimura, H., Meeuwsen, C.G., de Visser, H. Hazenberg, M.P., Wostmann, B.S., Pleasants, J.R. and Marcus, D.M. 1989. Eur. J. Immunol. 19: 2335.
17. Pereira, P., Forni, L., Larsson, E.-L., Cooper, M., Heusser, C. and Coutinho, A. 1986. Eur. J. Immunol. 16: 685.
18. Coffman, R.L., Seymour, B.W.P., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Schrader, B., Cherwinsky, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W. and Mosmann, T.R. 1988. Immunol. Rev. 102: 5.
19. Schwartz, R.H. 1985. Ann. Rev. Immunol. 3: 237.
20. Fultz, M., Finkelman, F.D. and Mond, J.J. 1984. J. Immunol. 133: 91.
21. Kruisbeek, A.M., Titus, J.A., Stephany, D.A., Gause, B.L. and Longo, D.L. 1985. J. Immunol. 134: 3605.
22. Kruisbeek, A.M., Fultz, M.J., Sharrow, S.O., Singer, A. and Mond, J.J. 1983. J. Exp. Med. 157: 1932.
23. Kruisbeek, A.M., Mond, J.J., Fowlkes, B.J., Carmen, J.A., Bridges, S. and Longo, D.L. 1985. J.

Exp. Med. 161: 1029.
24. Vos, Q., Klasen, E.A., and Haaijman, J.J. 1987. J. Immunol. Methods 103: 47.
25. Vos, Q. and Benner, R. 1989. J. Immunol. Methods 122: 43.

CHAPTER 3

SUBSTANTIALLY INCREASED SENSITIVITY OF THE SPOT-ELISA FOR THE DETECTION OF ANTI-INSULIN ANTIBODY-SECRETING CELLS USING A CAPTURE ANTIBODY AND ENZYME-CONJUGATED INSULIN*

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SUMMARY

This paper describes an antibody capture spot-ELISA for the detection of anti-insulin antibody-secreting cells. The assay is based on the binding of secreted antibodies by immobilised isotype-specific capture antibodies and subsequent detection of insulin-specific antibodies with a conjugate of human insulin and alkaline phosphatase (HI-AP). Compared with the conventional approach, using antigen for coating and employing an enzyme-linked detecting antibody, this technique improved the detection of cells secreting anti-insulin antibodies of the IgG-isotypes.

INTRODUCTION

Two types of assays can be used to enumerate antibody-secreting cells (ASC), namely haemolytic (Jerne and Nordin, 1963; Gronowicz et al., 1976) and reverse haemolytic (Eby et al., 1975) plaque assays and secondly, spot-ELISA (Sedgwick and Holt, 1983; Czerkinsky et al., 1983).

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Haemolytic plaque assay is limited by difficulties in coupling certain antigens to red cells (Golub et al., 1968; Pasanen and Mäkelä, 1969), instability of the products and the inability to determine the isotype of antibody contributing to direct plaque formation (Wortis et al., 1969). The sandwich spot-ELISA is more flexible and has been used for the simultaneous detection of distinct types of ASC (Czerkinsky et al., 1988b), enumeration of lymphokine producing lymphocytes (Czerkinsky et al., 1988a; Versteegen et al., 1988) and a variety of studies (Holt et al., 1984; Logtenberg et al., 1986; Bos et al., 1988; Zigterman et al., 1988).

In order to study the murine immune response to insulin we decided to develop a sandwich spot-ELISA for anti-insulin ASC, but found difficulty in detecting ASC of the IgG-isotypes. We adopted an antibody capture alternative approach (Clark and Adams, 1977; Crook and Payne, 1980; Vos and Benner, 1989) using a new method for coupling proteins to alkaline phosphatase (Claassen and Adler, 1988) to resolve the problem. This spot-ELISA allowed improved detection of cells secreting anti-insulin antibodies of the IgG-isotypes.

MATERIALS AND METHODS

Reagents

2-amino-2-methyl-1-propanol (AMP), alkaline phosphatase (AP), 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP), bovine serum albumin (BSA), glutaraldehyde (GA), lysine HCl and p-nitrophenyl phosphate were obtained from Sigma Chemical Co., St. Louis, MO. Isotype-specific goat anti-mouse (GAM) Ig antisera and antiserum alkaline phosphatase conjugates (GAM-AP) were from Southern Biotechnology, Birmingham, AL. Human semisynthetic monocomponent insulin (HI) was obtained from Novo Biolabs, Bagsvaerd, Denmark. Complete Freund's adjuvant (CFA) was from Difco, Detroit, MI. Glycerol and Tween 20 were from Merck, Darmstadt, F.R.G.. RPMI 1640 culture medium was from Gibco, Glasgow, Scotland. Monoclonal rat anti-mouse κ-light-chain (RAM-κ) (Yelton et al., 1981) and monoclonal mouse IgG1/κ anti-insulin p.10 (Vos and Benner, 1989) were purified by affinity chromatography. The protein content was determined by spectrophotometry at 280 nm.

Preparation of enzyme-conjugated insulin

A conjugate of HI and AP was prepared according to Claassen and Adler (1988). 5 mg AP were dissolved in 2 ml phosphate-buffered saline (PBS) and extensively dialysed against PBS. Next GA was added to the dialysis fluid to a

concentration of 0.2% and incubated overnight at 4°C. Excess GA was removed by dialysis of the reaction mixture against PBS and the activated AP was transferred to a test tube containing a solution of 5 mg HI in 4 ml PBS. After overnight incubation at 4°C, 0.25 ml 0.2 M lysine HCl were added, the mixture was incubated for 2 h at room temperature, purified by dialysis against PBS, filter-sterilized, diluted 1:1 with glycerol, and stored at -20°C.

Immunization of mice

Male BALB/c mice, 16 weeks of age, were immunized with 20 μg HI in 50 μl of an emulsion of PBS/CFA (1:1) in both rear footpads according to Schroer et al. (1979).

Preparation of cell suspensions

Cell suspensions from popliteal lymph nodes (PLN) were prepared as described by Benner et al. (1981). Concentrations of PLN cells and p.10 hybridoma cells (Vos and Benner, 1989) were determined with a Coulter Counter (Coulter Electronics, Luton, U.K.).

Sandwich ELISA

96 well plates (Titertek no. 77-172-05, Flow Laboratories, Zwanenburg, The Netherlands) were coated with 100 μl of a 10 μg/ml solution of HI in PBS by incubating for 1 h at 37°C. Next the plates were postcoated by adding 100 μl of PBS containing 1% BSA and 0.1% Tween 20 (PBT 1.0) to each well and incubating for 0.5 h at 37°C. After postcoating the plates were incubated with 100 μl of different concentrations of monoclonal antibody (MAb) p.10 in PBT 1.0. After 1 h of incubation at 37°C and three washes with PBS containing 0.1% BSA and 0.1% Tween 20 (PBT 0.1), 100 μl of a 1/1000 dilution of GAM-IgG_1-AP in PBT 1.0 were added, after which the plates were incubated for 1 h at 37°C. After five washes with PBT 0.1, 100 μl of substrate solution containing 0.2% PNP in AMP buffer (Sedgwick and Holt, 1983) were added. After 1 h of incubation at 37°C extinctions were determined spectrophotometrically (Titertek, Multiscan, Flow Laboratories, Irvine, U.K.).

Antibody capture ELISA

96 well plates were coated with 100 μl of a solution containing 10 μg/ml of GAM-IgG_1 in PBS. Next the plates were treated according to the sandwich ELISA protocol with exception of the detection of the MAb, for which 100 μl of a 1:100 dilution of HI-AP in PBT 1.0 were used instead of GAM-IgG_1-AP.

Sandwich spot-ELISA

96 well plates were coated with 100 μl of a solution of 10 μg/ml of HI in PBS by incubation for 1 h at 37°C. Next the plates were postcoated by adding 100 μl RPMI 1640 containing 1% BSA to each well and incubating for 0.5 h at 37°C. After postcoating, 100 μl of cell suspensions of different concentrations in RPMI 1640 containing 1% BSA were added and the plates were incubated for 6 h at 37°C in a vibration-free, 5% CO_2 incubator. Next the plates were washed three times with PBT 0.1 and 100 μl of a 1:1000 dilution of GAM-AP in PBT 1.0 were added to each well, after which the plates were incubated overnight at room temperature. After five washes with PBT 0.1, 100 μl of substrate solution containing 0.1% 5-BCIP in AMP buffer were added. After 1 h of incubation at 37°C the supernatants were discarded and the plates were washed three times with distilled water. The spots, identifiable by disposition of insoluble enzyme reaction product, were counted using a colony viewer (Bellco Glass, Vineland, NJ).

Antibody capture spot-ELISA

96 well plates were coated with 100 μl of solutions containing 10 μg/ml GAM or rat anti-mouse κ (RAM-κ) in PBS. Next the plates were treated according to the sandwich spot-ELISA protocol except for the detection of secreted Ig. Here plates coated with GAM received 100 μl of a 1/50 dilution of HI-AP in PBT 1.0 for detection of anti-insulin ASC, whereas plates coated with RAM-κ and incubated with 100 μl of a 1/1000 dilution of GAM-IgG_1-AP in PBT 1.0 were used to detect IgG1 IgSC.

RESULTS

In order to improve a sandwich spot-ELISA for anti-insulin ASC, which used HI for coating and GAM-AP for antibody detection, a spot-ELISA employing capture antibodies was developed. To detect insulin binding, a conjugate of HI and AP was prepared according to a method (Claassen and Adler, 1988) that ensured a minimal change in the immunogenic structure of the insulin molecule. An ELISA was used to compare the sensitivity of the capture-coating system with the sensitivity of the antigen-coating system. Fig. 1 shows the results from this comparison for the IgG_1/κ anti-insulin MAb p.10. Both titration curves show that the capture-system was approximately 25 times more sensitive than the system that employed antigen for coating. Results from the analysis of a panel of other anti-insulin MAb showed improvements of sensitivity that ranged from 10 to 100 times (data not shown).

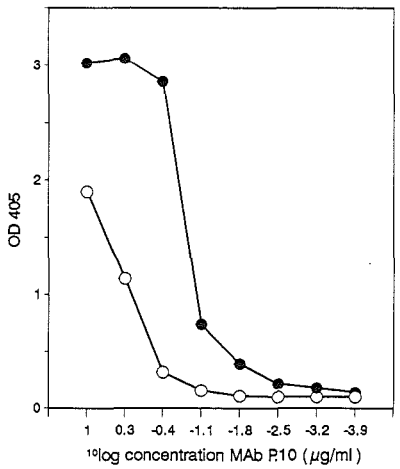

Figure 1. Titration curves of anti-insulin MAb p.10 by two types of ELISA. ○ : Plates were coated with HI and titrated with purified MAb. The detector antibody was GAM-IgG_1-AP. ● : Plates were coated with GAM-IgG_1 and titrated with purified MAb. HI-AP was used for detection.

Based on these results, the capture system was employed in an antibody capture spot-ELISA. Fig. 2 shows results from three types of spot-ELISA for p.10 hybridoma cells. Secretion of immunoglobulins was assayed by a system using RAM-κ for coating, and GAM-IgG_1-AP for detection.

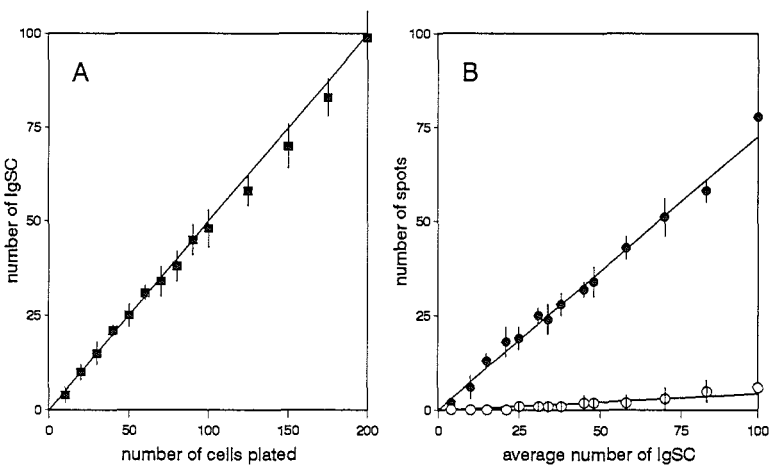

Figure 2. Spot-ELISA of p.10 anti-insulin hybridoma cells. **A**: detection of immunoglobulin secretion. Plates were coated with RAM-κ and various concentrations of cells were added. The detector antibody was GAM-IgG_1-AP. **B**: detection of anti-insulin ASC. ○ : plates were coated with HI and cells were added in different concentrations. The detector antibody was GAM-IgG_1-AP. ● : plates were coated with GAM-IgG_1 and various concentrations of cells were added. HI-AP was used for detection. Results are expressed as mean numbers ± SD (n=8).

Fig. 2A shows that this system detected immunoglobulin-secretion in 50% of the hybridoma cells. Fig. 2B shows the relative sensitivity of detection of HI-specific ASC by the sandwich and antibody capture spot-ELISAs. The data show that the antibody capture spot-ELISA detected approximately 75% of the immunoglobulin (IgSC), whereas the sandwich spot-ELISA, detected only 5%. Results from the analysis of a panel of anti-insulin hybridomas of IgG1 and IgG2 isotypes confirmed these percentages (data not shown).

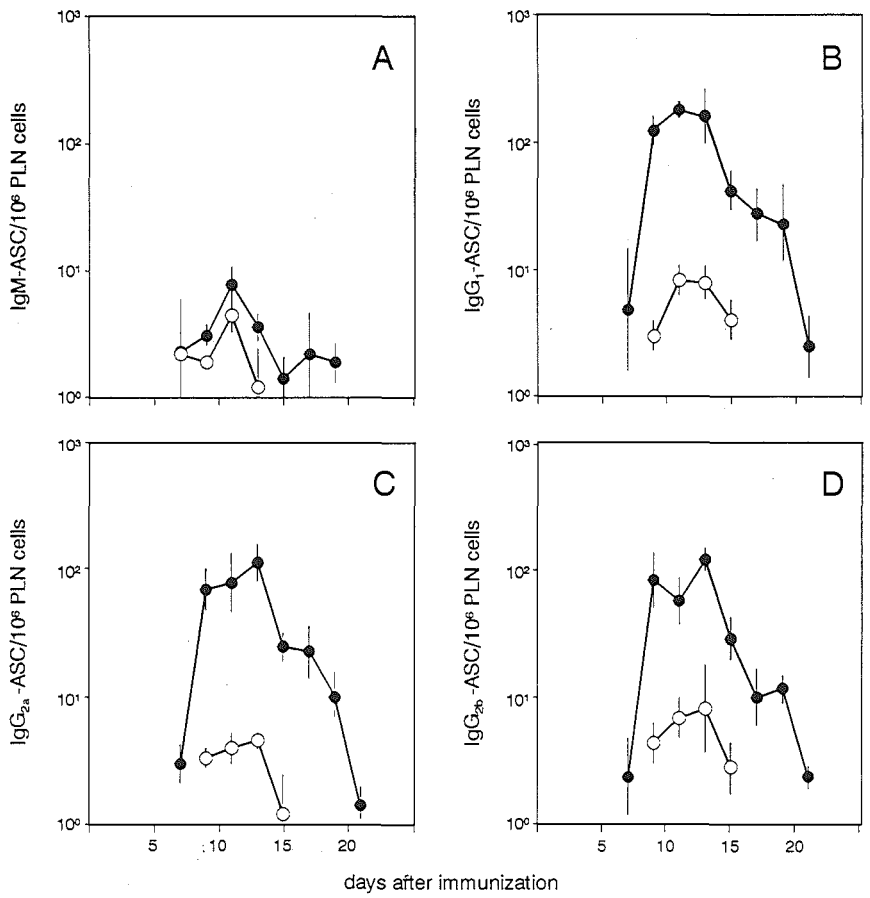

Figure 3. Kinetics of the primary anti-insulin ASC response in the popliteal lymph nodes of mice immunized with HI. Detection of ASC with two types of spot-ELISA. **A**: IgM ASC. **B**: IgG_1 ASC. **C**: IgG_{2a} ASC. **D**: IgG_3 ASC. ○ : plates were coated with HI and cells were added in different concentrations. The detector antibody was GAM-AP against the various isotypes. ● : plates were coated with GAM against the various isotypes and different concentrations of cells were added. HI-AP was used for detection. Results are expressed as geometric mean ± S.E.M. (n=3). During the first five days of the response no anti-insulin ASC could be detected. Results lower than 1 ASC/10^6 PLN-cells are not shown.

To test the new method on a polyclonal population of anti-insulin ASC, BALB/c mice were immunized with HI in CFA in both rear footpads. Popliteal lymph node (PLN) cells were isolated at various days after immunization and assayed for anti-insulin ASC. Fig. 3 shows the kinetics of the anti-insulin ASC response of the IgM, IgG_1, IgG_{2a} and IgG_{2b} isotypes assayed by the sandwich and the antibody capture spot-ELISA. These results illustrate the improvements that the use of a coating of capture antibodies and detection of secreted antibodies with an antigen-AP conjugate may bring on the conventional spot-ELISA for the detection of ASC of the IgG isotypes.

DISCUSSION

This paper describes a spot-ELISA for anti-insulin ASC. The use of capture antibodies to bind secreted antibodies and HI-AP to detect insulin binding resulted in a spot-ELISA with an improved sensitivity of ASC of the IgG isotypes. This improvement may be explained by an increase in the avidity of the plate-binding interaction, which will be greater for a dimeric IgG molecule than for a pentameric IgM. Moreover the sensitivity of the conventional spot-ELISA for IgM ASC may be attributed to the size of the IgM molecule, which can be simultaneously bound by a number of enzyme-linked second antibody-molecules.

The quality of the antigen-AP conjugate is crucial in the performance of the spot-ELISA based on capture antibodies. The conjugation method we used mildly activates the AP using glutaraldehyde (GA), resulting in a minimal inactivation and intermolecular crosslinking of the enzyme molecule. After removing excess of GA the antigen is conjugated under conditions of neutral pH and low salt concentration without the presence of organic solvents. These conditions make this method applicable for a large variety of protein-antigens.

The use of capture antibodies for coating allows this spot-ELISA to be employed for the enumeration of ASC of different antibody isotypes of various species. In order to have an optimal binding of secreted antibodies, the capture antibody should be as pure as possible. Purified MAb or affinity purified polyclonal antibodies are most suitable for this purpose. If a choice is to be made between different antibody preparations, an ELISA similar to the one described in Fig. 1 may be useful for performance testing.

The use of this spot-ELISA may facilitate studies of isotype-switching and other aspects of the kinetics of immune responses. Simultaneous detection of cells secreting antibodies of different specificities is another possibility of this spot-ELISA.

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REFERENCES

Benner, R., Van Oudenaren, A. and Koch, G. (1981) Induction of antibody formation in mouse bone marrow. In: I. Lefkovits and B. Pernis (Eds.), Immunological Methods. Academic Press, New York, Vol. 2, p. 247.

Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R. and Benner, R. (1988) The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. Cell. Immunol. 112, 371.

Claassen, E. and Adler, L.T. (1988) Sequential double immunocytochemical staining for in situ identification of an auto-anti-allotype immune response in allotype-suppressed rabbits. J. Histochem. Cytochem. 36, 1455.

Clark, M.F. and Adams, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475.

Crook, N.E. and Payne, C.C. (1980) Comparison of three methods of ELISA for baculoviruses. J. Gen. Virol. 46, 29.

Czerkinsky, C., Nilsson, L.-Å., Nygren, H., Ouchterlony, Ö. and Tarkowski, A. (1983) A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods 65, 109.

Czerkinsky, C., Andersson, G., Ekre, H.-P., Nilsson, L.-Å., Klareskog, L., Ouchterlony, Ö. (1988a) Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J. Immunol. Methods 110, 29.

Czerkinsky, C., Moldoveanu, Z., Mestecky, J., Nilsson, L.-Å., Ouchterlony, Ö. (1988b) A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody-secreting cells. J. Immunol. Methods 115, 31.

Eby, W.C., Chong, C.A., Dray, S. and Molinaro, G.A. (1975) Enumerating immunoglobulin-secreting cells among peripheral human lymphocytes. A hemolytic plaque assay for a B cell function. J. Immunol. 115, 1700.

Golub, E.S., Mishell, R.J., Weigle, W.O. and Dutton, R.W. (1968) A modification of the hemolytic plaque assay for use with protein antigens. J. Immunol. 100, 133.

Gronowicz, E., Coutinho, A. and Melchers, F. (1976) A plaque assay for all cells secreting Ig of a given type or class. Eur. J. Immunol. 6, 588.

Holt, P.G., Cameron, K.J., Stewart, G.A., Sedgwick, J.D. and Turner, K.J. (1984) Enumeration of human immunoglobulin-secreting cells by the ELISA-plaque method: IgE and IgG isotypes. Clin. Immunol. Immunopathol. 30, 159.

Jerne, N.K. and Nordin, A.A. (1963) Plaque formation in agar by single antibody-producing cells. Science 140, 405.

Logtenberg, T., Kroon, A., Gmelig-Meyling, F.H.J. and Ballieux, R.E. (1986) Production of anti-thyroglobulin antibody by blood lymphocytes from patients with autoimmune thyroiditis, induced by the insolubilized autoantigen. J. Immunol. 136, 1236.

Pasanen, V.J. and Mäkelä, O. (1969) Effect of the number of haptens coupled to each erythrocyte on haemolytic plaque formation. Immunology 16, 399.

Schroer, J.A., Inman, J.K., Thomas, J.W. and Rosenthal, A.S. (1979) H-2-linked Ir gene control of antibody responses to insulin. I. Anti-insulin plaque-forming cell primary responses. J. Immunol. 123, 670.

Sedgwick, J.D. and Holt, P.G. (1983) A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J. Immunol. Methods 57, 301.

Versteegen, J.M.T., Logtenberg, T. and Ballieux, R.E. (1988) Enumeration of IFN-γ-producing human lymphocytes by spot-ELISA. A method to detect lymphokine-producing lymphocytes at the single-cell level. J. Immunol. Methods 111, 25.

Vos, Q. and Benner, R. (1989) Analysis of anti-insulin antibodies using time-resolved fluoroimmunoassay J. Immunol. Methods 122, 43.

Wortis, H.H., Dresser, D.W. and Andersson, H.R. (1969) Antibody production studied by means of the localized haemolysis in gel (LHG) assay. III. Mouse cells producing five different classes of antibody. Immunology 17, 93.

Yelton, D.E., Desaymard, C. and Scharff, M. (1981) Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. Hybridoma 1, 5.

Zigterman, G.J.W.J., Verheul, A.F.M., Ernste, E.B.H.W., Rombouts, R.F.M., De Reuver, M.J., Jansze, M., Snippe, H. and Willers, J.M.N. (1988) Measurement of the humoral immune response against Streptococcus pneumoniae type 3 capsular polysaccharide and oligosaccharide containing antigens by ELISA and ELISPOT techniques. J. Immunol. Methods 106, 101.

CHAPTER 4

T CELL DEVELOPMENT IN GERMFREE BALB/c MICE FED A CHEMICALLY DEFINED ULTRAFILTERED DIET: DIFFERENTIAL DEPENDENCE OF T AND B CELL REPERTOIRES ON EXOGENOUS ANTIGENIC STIMULATION

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SUMMARY

The influence of exogenous antigenic stimulation on the development of T cells and the selection of their repertoire was investigated. For this purpose germfree BALB/c mice fed an ultrafiltered solution of chemically defined low molecular weight nutrients (GF-CD) were used. A comparison of the various lymphocyte populations in the thymus, lymph nodes and spleen from GF-CD and conventional mice using flow cytometry analysis revealed no significant differences. Flow cytometry analysis of the T cell receptor (TcR) Vβ expression on both mature thymocytes and lymph node T cells showed no differences between GF-CD and conventional mice. This stands in clear contrast to the B cell compartment, since the B cell repertoire of GF-CD mice has previously found to differ substantially from that of conventional mice. Activation of GF-CD splenic T cells by crosslinking with monoclonal antibodies (MAb) against TcR-$\alpha\beta$ resulted in proliferation both with and without addition of exogenous sources of lymphokines. These findings demonstrate that functional T cells develop in mice that have not been exposed to exogenous antigens and that the TcR repertoire, in contrast to the B cell compartment, is generated autonomously.

INTRODUCTION

T cells expressing TcR-$\alpha\beta$ recognize antigens presented in the context of self-major histocompatibility complex (MHC) molecules [1,2]. Since the TcR is generated by random rearrangement of DNA segments [3], the TcR repertoire must be shaped for appropriate MHC restriction and antigenic specificity. This process involves positive [4-12] and negative [13-19] selection in the thymus, both requiring interactions between TcR, MHC and accessory molecules like CD4 and CD8. The mature thymocytes evolving from these selective pressures can enter the periphery to display reactivity against non-self and tolerance to self antigens.

GF-CD mice have been used to study several aspects of B cell development and function in the absence of exogenous antigenic stimulation [20-27]. These studies showed that GF-CD mice had normal levels of serum IgM, but severely reduced levels of the other immunoglobulin (Ig) isotypes and corresponding frequencies of Ig secreting cells (IgSC) in spleen, lymph nodes and bone marrow. Moreover the usage of the Ig V_H-genes in hybridoma collections of adult GF-CD mice was found to be restricted and to resemble the repertoire of conventional neonatal mice, which is clearly different from the repertoire in adult conventional mice (26).

Based on a report on the presence of T cells in the peripheral lymphoid organs of GF-CD mice [28], we have investigated the development of T cells in animals free from exogenous antigenic stimulation. We have performed flow cytometry analysis on cells in thymus, lymph nodes and spleen of GF-CD mice and of expression of TcR-Vβ chains on mature thymocytes and lymph node T cells. Our findings suggest that the T cell repertoire of GF-CD mice, in contrast to the B cell repertoire, is similar to that of conventional mice. The proliferation of peripheral GF-CD T cells induced by TcR crosslinking indicated their functionality.

MATERIALS AND METHODS

Mice

GF-CD BALB/c mice, 8 to 10 weeks of age, were purchased from American Biogenetic Sciences (Notre Dame, IN). These mice were reared and maintained germfree in the Lobund Laboratory (University of Notre Dame, Notre Dame, IN) and fed a chemically defined ultrafiltered diet of low molecular weight nutrients consisting of L489-E14 Se and LADEK 69E6 [29]. Age-matched conventional BALB/c mice were obtained from the National Cancer Institute (Frederick, MD).

Preparation of cell suspensions

Suspensions of mature thymocytes were prepared as described previously [6,30]. Suspensions of purified splenic and lymph node T cells were prepared using anti A_β^d (MK-D6) [31] and anti E_α^d (14-4-4) [32] according to the same protocol.

Detection of IgSC

Splenic background IgSC in GF-CD and conventional mice were detected with the enzyme-linked immunosorbent assay (ELISA) plaque test [33] with some modifications described previously [34].

Flow cytometry

Cell suspensions were prepared in HBSS (without phenol red) containing 1% BSA and 0.1% sodium azide. Cells (10^6/100 μl buffer) were incubated on ice for 30 min with 10 μl of the appropriate antibody, and washed twice after each incubation. Control staining of cells with irrelevant antibody was used to obtain background fluorescence values. The samples were analyzed on a FACS 440 (Becton Dickinson & Co., Mountain View, CA) interfaced to a PDP 11/24 computer, as previously described [4]. Data were collected on 50,000 cells. Reagents used for direct staining were FITC- or biotin conjugated anti: CD3 (2C11) [35], CD4 (GK 1.5) [36], CD5 (53-7.3) [37], CD8 (2.43) [38], TcR-$\alpha\beta$ (H57-597) [39], Vβ3 (KJ25a) [40], Vβ5.1,5.2 (MR9-4) [41], Vβ6 (RR4-7) [42], Vβ8.1,8.2 (KJ16-133) [43,44], Vβ8.1,8.2,8.3 (F23.1) [47,48], Vβ11 (RR3-15) [46], I-A^d (MK-D6) [31], I-E^d (14-4-4) [32] and IgM (Becton Dickinson & Co.). Anti J11D [48] was used for indirect staining, employing anti-rat IgG as described [49].

T cell proliferation assay

For MAb induced T cell proliferation purified anti-TcR-$\alpha\beta$ (H57-597) [39] was diluted in PBS and 30 μl of solutions of various concentrations were added per round bottom microtiter well. Plates were incubated for 2 hours at 37°C and then washed 3 times with PBS before use. 2 x 10^5 T cell-enriched spleen cells in 200 μl complete DMEM (10% FCS, 5 x 10^{-5} M 2-ME, 10 mM Hepes, 200 mM glutamine, 1 mM sodium pyruvate, 10 U/ml penicillin and 100 μg/ml streptomycin) were added per well. Where indicated, recombinant human interleukin-2 (IL-2) was added at a final concentration of 10 U/ml. After 48 hours, cultures were pulsed with 1 μCi of [^{3}H]-thymidine and harvested 18 hours later. Values represent the arithmetic mean of triplicate cultures. Standard errors were generally less than 10% of the mean.

RESULTS

Frequencies of splenic background IgSC from GF-CD and conventional mice

Before considering the T cell development in the GF-CD mice, we confirmed earlier results on the B cell compartment in GF-CD mice. The frequencies of splenic background IgSC of various isotypes were determined employing the ELISA-plaque test (Fig. 1). As previously reported [20,24], the GF-CD mice had normal frequencies of IgM-SC, but highly reduced numbers of cells secreting the other isotypes. Thus, the B cell compartment is severely affected by the absence of exogenous antigens.

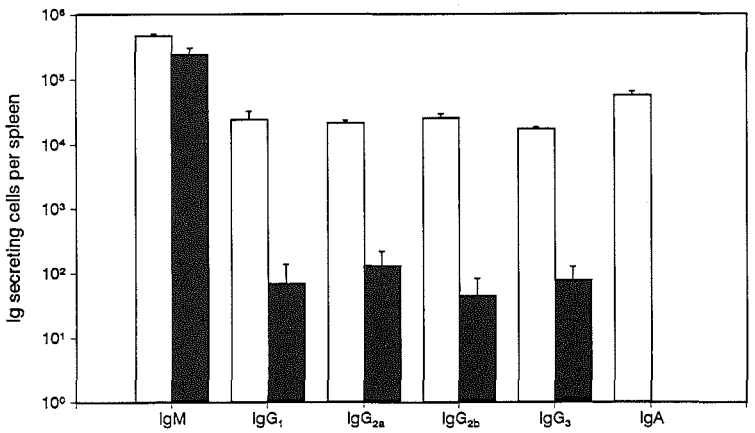

Figure 1. Frequency of background IgSC of various isotypes in the spleens of GF-CD (solid columns) and conventional mice (open columns). The data are expressed as the geometric mean of three individually tested animals ± SEM.

Comparison of thymocyte populations from GF-CD and conventional mice

Table 1 summarizes the results from the flow cytometry analysis of thymocyte populations of GF-CD and conventional mice. The GF-CD mice had similar numbers of CD4 and CD8 single-positive thymocytes as well as of double-positive and double-negative cells as conventional mice. Analysis with anti-CD5 also indicated no significant differences among the major thymocyte populations [50]. The staining of thymocytes from GF-CD and conventional mice for the HSA marker indicated comparable frequencies of immature J11D$^+$ cells [55]. Staining with anti-TcR, -CD4 and -CD8 yielded similar results for both GF-CD and conventional mice as did the analysis with

anti-TcR and anti-CD3, with the population of $CD3^+TcR\text{-}\alpha\beta^-$ cells representing the population of $TcR\text{-}\gamma\delta^+$ thymocytes.

TABLE 1. Flow cytometry analysis of lymphocyte populations in the thymus of GF-CD and conventional mice

Population	GF-CD	Conventional
$CD4^+CD8^+$	73.1	76.8
$CD4^+CD8^-$	11.9	10.7
$CD4^-CD8^+$	4.3	5.9
$CD4^-CD8^-$	10.7	6.7
$CD8^+CD5^+$	80.8	79.9
$CD8^-CD5^+$	12.0	11.9
$J11D^+CD4^+$	73.8	71.1
$J11D^+CD8^+$	65.4	68.0
$CD4^+TcR\text{-}\alpha\beta^+$	14.3	14.9
$CD4^+TcR\text{-}\alpha\beta^-$	74.6	77.1
$CD8^+TcR\text{-}\alpha\beta^+$	8.0	7.8
$CD8^+TcR\text{-}\alpha\beta^-$	74.0	76.6
$CD3^+TcR\text{-}\alpha\beta^-$	2.4	2.0

Numbers represent the percentages of positive cells and are representative of 3 separate experiments with pooled thymuses from 3 or 4 mice.

In order to study the repertoire of TcR Vβ chains expressed on the mature thymocytes, thymic cell suspensions were enriched for single-positive T cells by treatment with anti-J11D MAb and complement. The resulting populations from both GF-CD and conventional mice were analyzed for Vβ expression with a panel of MAb specific for different TcR Vβ chains. The data of this analysis are summarized in Table 2. The analysis indicates no significant differences between the repertoires of mature thymocytes of GF-CD and conventional mice. The reduced percentages of cells with Vβ3, Vβ5 and Vβ11 indicate that GF-CD thymocytes are subject to a normal clonal deletion for reactivity with superantigens [40] and peptides in association with $E_\alpha E_\beta$

[45,46], respectively.

TABLE 2. Flow cytometry analysis of TcR-Vβ expression on mature thymocytes from GF-CD and conventional mice

TcR	GF-CD		Conventional	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Vβ2	10.0[a]	4.4[b]	12.9	6.7
Vβ3	0.3	0.0	0.4	0.8
Vβ5.1	0.7	0.7	0.6	0.0
Vβ5.1+5.2	0.4	1.3	0.6	0.4
Vβ6	7.1	7.6	6.4	5.7
Vβ8.1+8.2	32.4	22.2	39.8	26.5
Vβ8.1+8.2+8.3	34.0	32.1	39.1	37.4
Vβ11	0.5	1.0	1.3	0.0

a. Numbers represent percentages of the population of mature thymocytes carrying CD4 and TcR-$\alpha\beta$ and are representative of 3 separate experiments with pooled thymuses from 3 or 4 mice. Enrichment was obtained by treatment with J11D plus complement as described [6].

b. Numbers represent percentages of the population of mature thymocytes carrying TcR-$\alpha\beta$ and negative for CD4; reciprocal staining confirmed the CD8 phenotype of these cells.

Comparison of lymph node cell populations from GF-CD and conventional mice

Table 3 shows the results from a flow cytometry analysis of lymph node cells from GF-CD and conventional mice. The distribution of $CD4^+$, $CD8^+$ and $CD3^+$ T cells and surface Ig (sIg) positive B cells were very similar as were the percentages of I-A and I-E bearing cells. For the analysis of the TcR Vβ chain expression, lymph node cells were treated with a mixture of anti-A_β and anti-E_α MAbs and complement and were stained according to the same protocol as the mature thymocytes. The resulting data are summarized in Table 4. The results indicate a high degree of similarity between the Vβ repertoires of the lymph node T cells of GF-CD and conventional mice.

TABLE 3. Flow cytometry analysis of lymphocyte populations in lymph nodes from GF-CD and conventional mice

Population	GF-CD	Conventional
CD4	44.7	46.2
CD8	18.1	19.0
sIg	38.2	34.3
I-A	34.9	34.2
I-E	20.9	19.4

Numbers in the columns represent the percentage of positive cells and are representative of 3 separate experiments with pooled lymph nodes from 3-4 mice.

TABLE 4. Flow cytometry analysis of TcR-Vβ expression on purified lymph node T cells from GF-CD and conventional mice

TcR	GF-CD		Conventional	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Vβ2	6.9[a]	8.8[b]	6.8	7.5
Vβ3	0.3	0.6	0.3	0.7
Vβ5.1	0.2	2.7	0.3	3.0
Vβ5.1+5.2	0.3	3.2	0.2	3.1
Vβ6	12.0	14.2	12.3	14.7
Vβ8.1+8.2	11.5	15.2	11.6	13.7
Vβ8.1+8.2+8.3	18.3	30.8	19.9	29.0
Vβ11	0.2	2.1	0.3	2.6

a. Numbers represent percentages from the population of purified T cells carrying CD4 and TcR-$\alpha\beta$; T cells were purified by treatment with anti-A_β and anti-E_α plus complement as described and collected from pooled lymph nodes from 3 mice.

b. Numbers represent percentages from the population of purified T cells carrying TcR-$\alpha\beta$ and negative for CD4; reciprocal staining confirmed the CD8 phenotype of these cells.

Comparison of splenic cell populations from GF-CD and conventional mice

Table 5 shows the results from a flow cytometry analysis of splenocytes from GF-CD and conventional mice. The data again indicate no significant differences and confirm the presence of MHC class II molecules on a fraction of the GF-CD lymphocytes.

TABLE 5. Flow cytometry analysis of splenic lymphocyte population from GF-CD and conventional mice

Population	GF-CD	Conventional
CD4	31.8	27.6
CD8	14.9	13.3
sIg	49.4	46.2
I-A	46.6	45.6
I-E	32.2	34.8

Numbers in the columns represent the percentages of positive cells and are representative of 3 separate experiments with pooled spleens from 3 or 4 mice.

In vitro activation of splenocytes from conventional and GF-CD mice

The previous data indicated the presence of normal frequencies of T cells in the peripheral lymphoid organs of the GF-CD mice and a normal TcR Vβ repertoire. In order to investigate whether these T cells were also functional, spleen cells were stimulated *in vitro* by MAb against TcR-$\alpha\beta$ with or without the addition of IL-2. Fig. 2 shows the results from the stimulation of purified splenic T cells from both GF-CD and conventional mice. The data indicate that the splenic T cells were induced to proliferation by TcR crosslinking, showing that GF-CD mice had functional peripheral T cells.

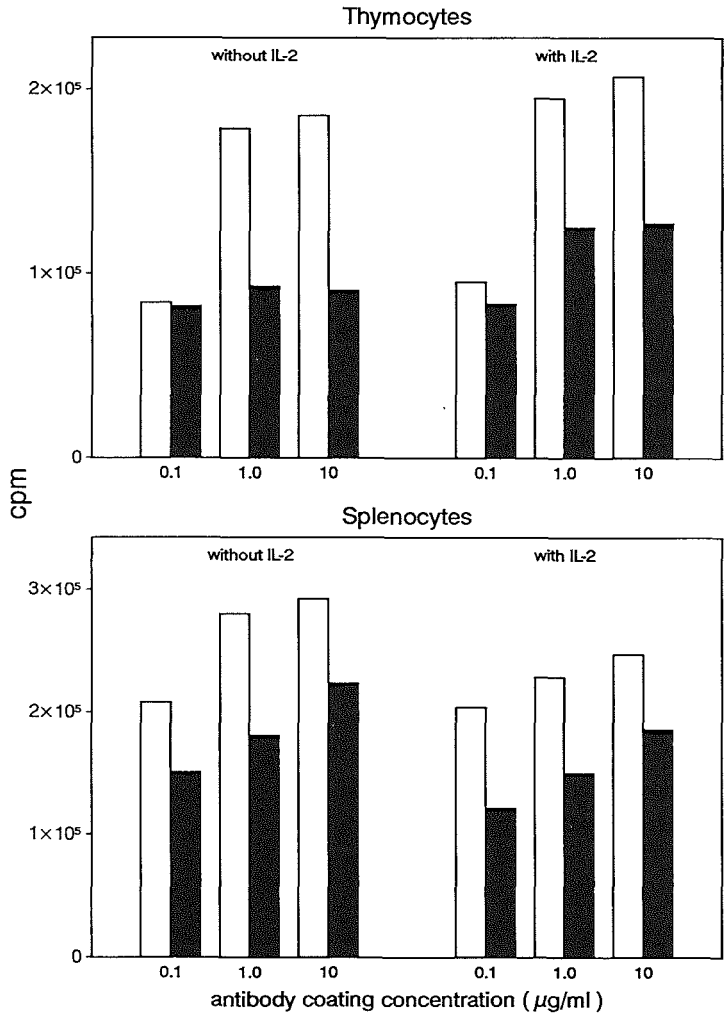

Figure 2. *In vitro* stimulation of purified splenic T cells from GF-CD (solid columns) and conventional mice (open columns). 2 x 10^5 T cell enriched spleen cells were cultured in presence or absence of added IL-2 in microtiter wells coated with various concentrations of anti-TcR-$\alpha\beta$ antibodies.

DISCUSSION

In this paper we have investigated the development of T cells in mice that had never been exposed to exogenous antigenic stimulation. This implies that endogenous self-antigens were the only source of MHC bound peptides [52] involved in both thymic development and peripheral T cell selection. Previous data on the development of B cells in GF-CD mice have shown that the frequency of IgSC of non-IgM isotypes was highly reduced. These findings suggest that the T cell repertoire is shaped in such a way by negative selection, that mature T cells cannot be induced by endogenous antigens to secrete T cell factors that can induce isotype switching [53] in B cells.

The presence of functional T cells in the spleen and lymph nodes of GF-CD mice supports evidence from radiation chimaera experiments on the role of self-peptides in positively selecting the T cell repertoire [54].

From this and the demonstration of MHC class II molecules on lymphocytes in the peripheral lymphoid organs it could be speculated that functional T cells indeed recognize the presented self-peptides, but that thymic selection has shaped their repertoire in such a way that these encounters do not induce differentiation into T_{H0} cells [55] capable of producing interferon γ and interleukin 4.

The high extent of similarity between the Vβ repertoires of mature thymocytes from GF-CD and conventional mice and especially the equal presence of cells carrying Vβ8.1,8.2 and 8.3 indicate [56] that exogenous superantigens like staphylococcal enterotoxin B only have a marginal influence on the selection of the T cell repertoire. The results from this Vβ repertoire analysis, however, do not allow a definitive conclusion on the similarity between TcR repertoires of GF-CD and conventional mice. For this purpose a thorough analysis of the N-junctional diversity in the TcR repertoire of these mice should be performed, since this mechanism of TcR gene assembly is of great importance for the generation of a diverse TcR-repertoire (57).

Based on the analysis of the size distribution of T and B lymphocytes it has been concluded [28] that GF-CD mice possess activated T and B cells. The question of the state of activation of peripheral T cells in GF-CD mice is of great importance for our understanding of T cell function in GF-CD and conventional animals. Our results allow no direct answer to this question and we believe that supplementary experiments need to be performed to conclude the presence of activated T cells in GF-CD mice.

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REFERENCES

1. Zinkernagel, R.M. and Doherty, P.C. Nature 1974. 251:547.
2. Davis, M.M. and Bjorkman, P.J. Nature 1988. 334:395.
3. Arden, B., Klotz, J.L., Siu, G. and Hood, L.E. Nature 1985. 316:783.
4. Kruisbeek, A.M., Mond, J.J., Fowlkes, B.J., Carmen, J.A., Bridges, S. and Longo, D.L. J. Exp. Med. 1985. 161:1029.
5. Marušić-Galesić, S., Stephany, D., Longo, D.L. and Kruisbeek, A.M. Nature 1988. 333:180.
6. Zuñiga-Pflücker, J.C., Longo, D.L. and Kruisbeek, A.M. Nature 1989. 338:76.
7. Teh, H.S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H. and von Boehmer, H. Nature 1988. 335:229.
8. Kisielow, P., Teh, H.S., Blüthmann, H. and von Boehmer, H. Nature 1988. 335:730.
9. Scott, B., Blüthmann, H., Teh, H.S. and von Boehmer, H. Nature 1989. 338:591.
10. MacDonald, H.R., Lees, R.K., Schneider, R., Zinkernagel, R.M. and Hengartner, H. Nature 1988. 336:471.
11. Berg, L.J., Pullen, A.M., Fazekas de St.Groth, B., Mathis, D., Benoist, C. and Davis, M.M. Cell 1989. 58:1035.
12. Blackman, M.A., Marrack, P. and Kappler, J. Science 1989. 244:214.
13. Kappler, J.W., Roehm, N. and Marrack, P. Cell 1987. 49:273.
14. Kappler, J.W., Staerz, U., White, J. and Marrack, P.C. Nature 1988. 332:35.
15. MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M. and Hengartner, H. Nature 1988. 332:40.
16. Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. Nature 1988. 333:742.
17. Sha, W.C., Nelson, C.A., Newberry, R.D., Kranz, D.M., Russell, J.H. and Low, D.Y. Nature 1988. 336:73.
18. Hodes, R.J., Sharrow, S.O. and Solomon, A. Science 1989. 246:1041.
19. Fry, A.M., Jones, L.A., Kruisbeek, A.M. and Matis, L.A. Science 1989. 246:1044.
20. Hooijkaas, H., Benner, R., Pleasants J.R. and Wostmann, B.S. Eur. J. Immunol. 1984. 14:1127.
21. Hooijkaas, H., van der Linde-Preesman, A.A., Bitter, W.M., Benner, R. Pleasants, J.R. and Wostmann, B.S. J. Immunol. 1985. 134:2223.
22. Bos, N.A., Benner, R., Wostmann, B.S. and Pleasants, J.R. J. Reprod. Immunol. 1986. 9:237.
23. Bos, N.A. Meeuwsen, C.G., Hooijkaas, H., Benner, R., Wostmann, B.S. and Pleasants, J.R. Cell. Immunol. 1987. 105:235.
24. Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R. and Benner, R. Cell. Immunol. 1988. 112:371.
25. Bos, N.A. and Meeuwsen, C.G. Eur. J. Immunol. 1989. 19:1811.
26. Bos, N.A., Meeuwsen, C.G., van Wijngaarden, P. and Benner, R. Eur. J. Immunol. 1989. 19:1817.
27. Bos, N.A., Kimura, H., Meeuwsen, C.G., de Visser, H., Hazenberg, M.P., Wostmann, B.S., Pleasants, J.R., Benner, R. and Marcus, D.M. Eur. J. Immunol. 1989. 19:2335.
28. Pereira, P., Forni, L., Larsson, E.-L., Cooper, M., Heusser, C. and Coutinho, A. Eur. J. Immunol.

1986. 16:685.
29. Pleasants, J.R., Johnson, M. and Wostmann, B.S. J. Nutr. 1986. 116:1949.
30. Zuñiga-Pflücker, J.C., McCarthy, S.A., Weston, M., Longo, D.L., Singer, A. and Kruisbeek, A.M. J. Exp. Med. 1989. 169:2085.
31. Kappler, J.W., Skidmore, B., White, J. and Marrack, P. J. Exp. Med. 1981. 153:1198.
32. Ozato, K., Mayer, N. and Sachs, D.H. J. Immunol. 1980. 124:533.
33. Sedgwick, J.D. and Holt, P.G. J. Immunol. Methods 1983. 57:301.
34. Vos, Q., Claassen, E. and Benner, R. J. Immunol. Methods 1990. 126:89.
35. Leo, O., Foo, M., Sachs, D.H., Samelson, L.E. and Bluestone, J.A. Proc. Natl. Acad. Sci. USA 1987. 84:1374.
36. Dialynas, D.P., Quan, Z.S., Wall, K.A., Pierres, A., Quintans, J., Loken, M.R., Pierres, M. and Fitch, F.W. J. Immunol. 1983. 131:2445.
37. Ledbetter, J.A. and Herzenberg, L.A. Immunol. Rev. 1979. 47:63.
38. Sarmiento, M., Glasebrook, A.L. and Fitch, F.W. J. Immunol. 1980. 125:2665.
39. Kubo, R.T., Born, W., Kappler, J.W., Marrack, P. and Pigeon, M. J. Immunol. 1989. 142:2736.
40. Pullen, A.M., Marrack, P. and Kappler, J.W. Nature 1988. 335:796.
41. Woodland, D., Kapp, M.P., Bill, J. and Palmer, E. Science 1990. 247:964.
42. Kanagawa, O., Palmer, E. and Bill, J. Cell. Immunol. 1989. 119:412.
43. Haskins, K., Hannum, C., White, J., Roehm, N., Kubo, R., Kappler, J. and Marrack, P. J. Exp. Med. 1984. 160:452.
44. Behlke, M.A., Henkel, T.J., Anderson, S.J., Lan, N.C., Hood, L., Braciale, V.L., Braciale, T.J. and Loh, D.Y. J. Exp. Med. 1987. 165:257.
45. Staerz, U.D., Rammensee, H.-G., Benedetto, J.D. and Bevan, M.J. J. Immunol. 1985. 134:3994.
46. Bill, J., Kanagawa, O., Woodland, D.L. and Palmer, E. J. Exp. Med. 1989. 169:1405.
47. Liao, N.-S., Maltzman, J. and Raulet, D.H. J. Exp. Med. 1989. 170:135.
48. Bruce, J., Symington, F.W., McKearn, T.J. and Sprent, J. J. Immunol. 1981. 127:2496.
49. McCarthy, S.A., Kruisbeek, A.M., Uppenkamp, I.K., Sharrow, S.O. and Singer, A. Nature 1988. 336:76.
50. Fowlkes, B.J., Edison, L., Mathieson, B.J. and Chused, T.M. J. Exp. Med. 1985. 162:802.
51. Crispe, I.N. and Bevan, M. J. Immunol. 1987. 138:2013.
52. Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. Nature 1987. 329:506.
53. O'Garra, A., Umland, S., De France, T. and Christiansen, J. Immunol. Today 1988. 9:45.
54. Nikolić-Zugić, J. and Bevan, M.J. Nature 1990. 344:65.
55. Firestein, G.S., Roeder, W.D., Laxer, J.A., Townsend, K.A., Weaver, C.T., Hom, J.T., Linton, J., Torbett, B.E. and Glasebrook, A.L. J. Immunol. 1989. 143:518.
56. White, J., Herman, A., Pullen, A.M., Kubo, R., Kappler, J.W. and Marrack, P. Cell 1989. 56:27.
57. Kronenberg, M., Sui, G., Hood, L.E. and Shastri, N. Ann. Rev. Immunol. 1986. 4:529.

CHAPTER 5

AUTONOMOUS T CELL ACTIVATION IN GERMFREE MICE FED A CHEMICALLY DEFINED LOW MOLECULAR ULTRAFILTERED DIET

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SUMMARY

Germfree mice fed a chemically defined low molecular weight ultrafiltered diet (GF-CD) were used to study the functionality of T cells generated in an environment free from exogenous antigenic stimulation. Results from mixed lymphocyte cultures and antibody mediated T cell receptor crosslinking indicated that GF-CD T cells were capable of mounting normal alloreactive responses and of secreting lymphokines that could induce isotype switching in GF-CD B cells. Flow cytometry analysis indicated the presence of the IL-2 receptor on GF-CD splenic T cells. Limiting dilution analysis of purified GF-CD splenic T cells indicated the presence of the high affinity IL-2 receptor, suggesting the presence of activated T cells. The significance of these findings for the internal activity of the normal immune system and its function are discussed.

INTRODUCTION

The immune system is indispensable in the defense of the vertebrate host to a variety of infectious agents. By defining the immune system as a host defense machinery against non-self, the distinction between self and non-self becomes a main feature of its function. To prevent a "horror autotoxicus" (1), a state of self-tolerance should be maintained. Three mechanisms have been proposed (2) preventing T cell

autoreactivity: clonal deletion (3), clonal anergy (4) and suppression (5). Autoimmunity is generally considered to be caused by a failure of one or more of these mechanisms (6). The demonstration of selfreactive clones among mature lymphocytes in several experimental *in vitro* systems (7-9), however, suggests that T cells with autoreactive properties may be present in the peripheral organs of animals not suffering from autoimmune disorders. These findings support hypotheses that self-recognition is essential within the immune system (10-11) and that host-defense is a function of the immune system, but not its "raison d'être" (12,13).

Studies using non-intentionally immunized animals have indicated background activity in the immune system (14,15), but cannot exclude the influence of bacteria, viruses and other causes of exogenous antigenic stimulation on these results. The availability of germfree mice fed a chemically defined ultrafiltered low molecular weight diet (GF-CD) offers the opportunity to study the immune system of animals that have lived in the complete absence of exogenous antigenic stimulation. Studies on the B cell development in GF-CD mice (16-19) have shown a highly reduced number of cells secreting immunoglobulins (Ig) of the non-IgM isotypes, a restricted use of Ig-V_H genes and a high extent of multireactivity within the pool of lipopolysaccharide (LPS)-reactive B cells. Studies on the development of T cells in GF-CD mice (20,21) have indicated a normal incidence and repertoire of mature thymocytes and a normal repertoire of non-anergic peripheral T cells. In the present study we have investigated the functional properties of peripheral T cells from GF-CD mice, showing their ability to mount allogeneic responses and to produce lymphokines that could induce isotype switching in GF-CD B cells. Using flow cytometry analysis the interleukin 2 receptor (IL-2R) could be detected on a significant part of splenic GF-CD T cells. Limiting dilution analysis of GF-CD splenic T cells on IL-2 indicated the presence of cells carrying the high affinity IL-2R. These findings suggest the presence of activated T cells in GF-CD mice and support hypotheses on an autonomous activation of the immune system.

MATERIALS AND METHODS

Mice

GF-CD BALB/c mice, 8 to 10 weeks of age, were purchased from American Biogenetic Sciences (Notre Dame, IN). These mice were reared and maintained germfree in the Lobund Laboratory (University of Notre Dame, Notre Dame, IN) and fed a chemically defined ultrafiltered diet of low molecular weight nutrients consisting of L489-E14 Se and LADEK 69E6 (22). Age-matched specific pathogen free (SPF) BALB/c mice and conventional BALB/c, C57BL/6, C3H and DBA/2 mice were

obtained from the National Cancer Institute (Frederick, MD).

Tissue culture medium

DMEM supplemented with penicillin at 10 U/ml, streptomycin at 100 μg/ml, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 10% heat inactivated fetal calf serum was used for all cell cultures.

T cell purification

T cells were purified from splenocyte suspensions by treatment with anti-A_β^d, MK-D6 (23), anti-E_α^d, 14-4-4 (24) and complement.

Mixed lymphocyte culture

The indicated number of purified splenic T cells were incubated with 5 x 10^5 irradiated (2000 rads) spleen cells in 96-well flat-bottom microtiter plates. After 3 days, cultures were pulsed with 1 μCi of [^{3}H]-thymidine and harvested 18 hours later. All determinations were performed in triplicate. Data are expressed as Δcpm i.e., cpm values of experimental groups minus responder cells alone.

***In vitro* stimulation with anti-CD3**

15 ml culture flasks were coated with 50 μg/ml purified anti-CD3, 2C11 (25) in PBS by incubation for 2 hours at 37°C and washed once with tissue culture medium. Next 2 x 10^7 splenocytes in 2 ml medium were added to anti-CD3 or control (PBS coated flasks) and incubated for the indicated time. After culture, cells were removed and counted with a hemocytometer.

ELISA spot assays

Interferon-γ (IFN-γ) secreting cells were detected as described (26), Ig-secreting cells (IgSC) of various isotypes according to Sedgwick and Holt (27) with some modifications (28).

Flow cytometry analysis

Cell suspensions were prepared and analysed as described previously (29). Reagents used for staining were fluorescein isothiocyanate-conjugated anti-IL-2R,

PC.61 (30), biotin-conjugated anti-CD4, GK 1.5 (31), and anti-T cell receptor(TcR)-$\alpha\beta$, H57-597 (32).

Limiting dilution analysis of high affinity IL-2R expression

Multiple cultures of various concentrations of purified splenic T cells were cultured in 96-well round bottom microtiter plates with or without the addition of IL-2 at a final concentration of 10 U/ml. After 3 days, cultures were pulsed with 1 μCi of [^{3}H]-thymidine and harvested 18 hours later. Cultures containing IL-2 were scored positive if their counts exceeded the arithmetic mean plus 3 SD of cultures of corresponding numbers of cells grown in the absence of IL-2.

RESULTS

Mixed lymphocyte cultures of GF-CD splenic T cells

In order to investigate the response of GF-CD T cells to a stimulus involving TcR mediated recognition of major histocompatibility complex (MHC) molecules and associated antigens, BALB/c GF-CD splenic T cells were stimulated with allogeneic spleen cells. Spleen cells used for stimulation were from C3H (H-2^b, Mls^c), C57BL/6 (H-2^b, Mls^b), DBA/2 (H-2^d, Mls^a) and, as a control, BALB/c (H-2^d, Mls^c). Fig. 1 shows the results from a comparison of the responses of three doses of T cells from GF-CD and conventional BALB/c mice. The data indicate that GF-CD T cells can mount the same responses as conventional mice, with a comparable dose-response relationship, both after stimulation by cells of a different MHC haplotype, C57BL/6 and C3H, and to MHC-compatible cells presenting a different minor lymphocyte stimulating (Mls) antigen, DBA/2. These findings show that GF-CD T cells can be activated by recognition of allogeneic MHC molecules and associated antigens via the TcR. BALB/c GF-CD T cells, however, did not respond to cells from conventional BALB/c mice.

In vitro stimulation test for lymphokine secretion by GF-CD spleen cells

Studies on the B cell development in GF-CD mice have shown a highly reduced number of cells secreting Ig of the non-IgM isotypes (16,17) and have indicated that mitogenic stimulation could induce isotype switching in GF-CD splenic B cells (33). The presence of T cells with functional capabilities in combination with a highly reduced number of cells secreting Ig of "switched" isotypes could indicate that either GF-CD T cells were not able to secrete lymphokines after stimulation, GF-CD B cells were not

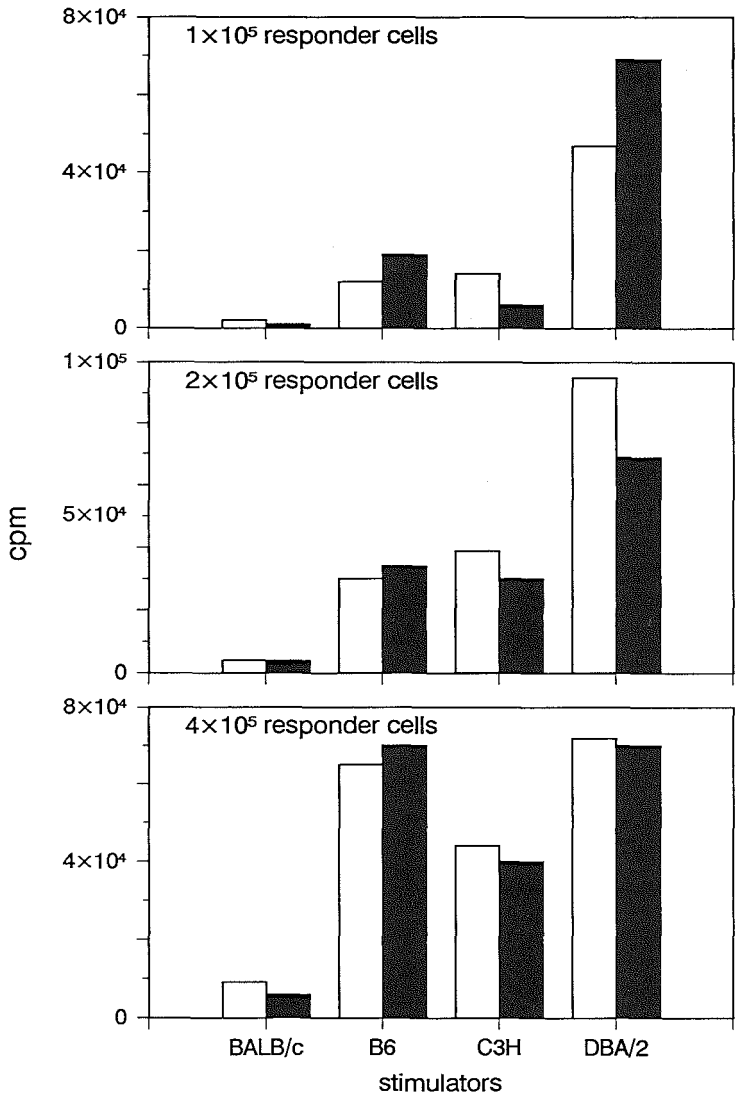

Figure 1. Allogeneic responses of different doses of splenic T cells from GF-CD (solid columns) and conventional (open columns) BALB/c mice to irradiated spleen cells from various donors.

capable of responding to secreted lymphokines by isotype switching or that repertoire selection prevented T cells from lymphokine secretion induced by self-antigens. To test these hypotheses, high density cultures of 2 x 10^7 spleen cells in 2 ml medium were set up in 15 ml culture flasks coated with anti-CD3 antibody, to induce TcR complex crosslinking. Simultaneous cultures were set up with GF-CD and SPF BALB/c spleen cells in anti-CD3 and control coated culture flasks. Cells were harvested at days 0, 1, 3, 4 and 5, counted and assayed for cells secreting IFN-γ and Ig of various isotypes. Figs. 2 and 3 show the results from these tests. Fig. 2a indicates a marked difference in the percentage of GF-CD cells surviving in anti-CD3 stimulated and unstimulated cultures. The results from SPF spleen cells were very similar. Fig. 2b shows the secretion of IFN-γ by anti-CD3 stimulated GF-CD and SPF spleen cells and the development of IFN-γ secreting spleen cells in cultures not stimulated with anti-CD3.

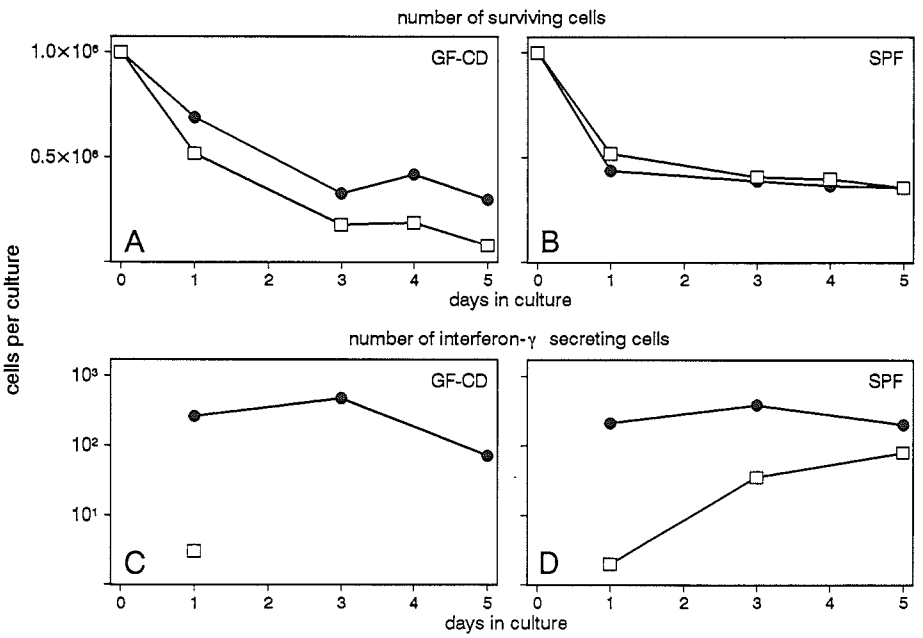

Figure 2. *In vitro* stimulation of spleen cells from GF-CD and SPF BALB/c mice by anti-CD3 crosslinking. ● : stimulated cultures, □ : control cultures. Responses represent numbers of surviving cells and numbers of IFN-γ secreting cells.

Fig. 3 shows the development of IgSC of various isotypes after *in vitro* stimulation. The data indicate that *in vitro* stimulation of GF-CD spleen cells can induce the development of cells secreting Ig of non-IgM isotypes. The presence of cells secreting IgG1,

IgG2a and IgA indicate the secretion of IL-4, IFN-γ and IL-5, respectively (34). These findings show that GF-CD T cells can be induced to secrete various lymphokines and that GF-CD B cells are able to respond to these factors by isotype switching. On basis of these results the marginal frequency of cells secreting Ig other than IgM is most likely the consequence of negative selection (3,35,36) of the T cell repertoire for cells displaying autoreactivity resulting in lymphokine secretion.

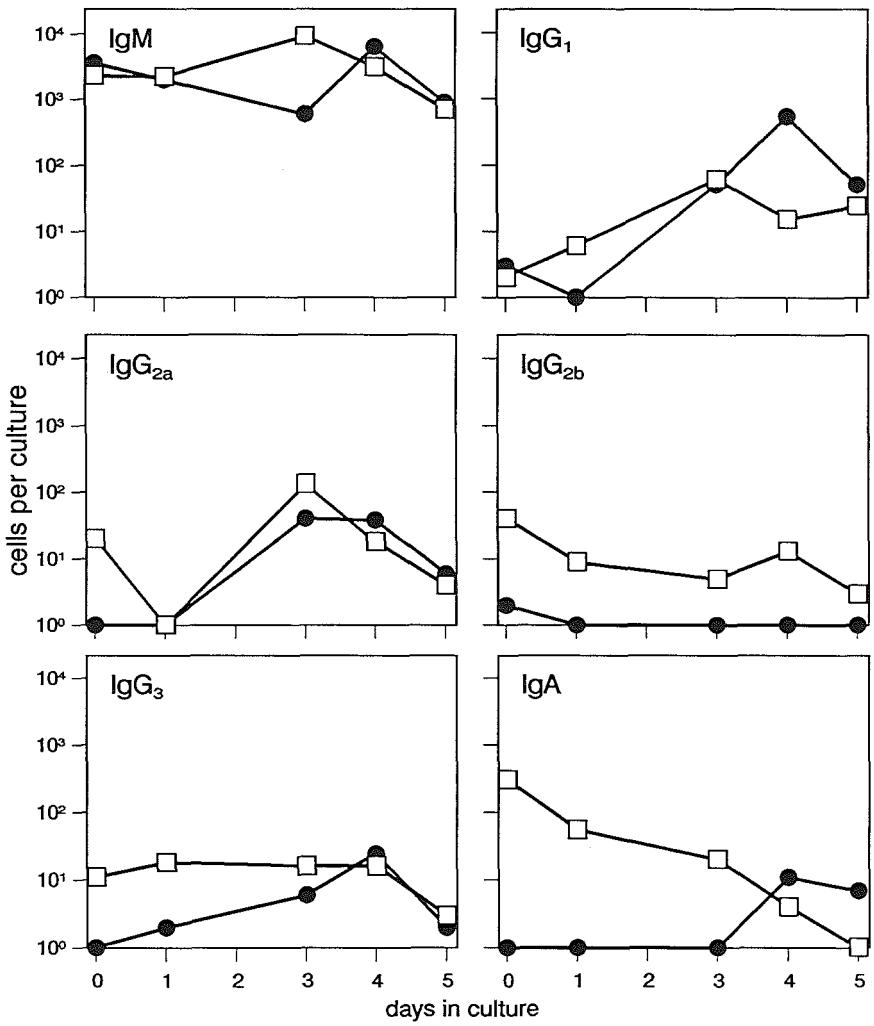

Figure 3. *In vitro* stimulation of spleen cells from ● : GF-CD and □ : SPF BALB/c mice by anti-CD3 crosslinking. Responses represent numbers of IgSC of various isotypes.

FACS analysis of IL-2R expression on GF-CD spleen and lymph node cells

Besides negative selection, positive selection (37,39) is considered an important process in the maturation of thymocytes. The presence of functional T cells in GF-CD mice supports evidence from radiation chimaera experiments (40), that suggested the role of self-peptides in the positive selection of the T cell repertoire. Thus it could be speculated that GF-CD T cells recognize self-antigens in the peripheral lymphoid organs, but that their response to these stimuli is of a limited nature. A way to test this hypothesis is to analyze the activational state of T cells in GF-CD mice. For this purpose spleen and lymph node cells from GF-CD mice were analyzed for expression of the IL-2R, a marker for activated T cells (41,42). Table 1 summarizes the data of the FACS analysis. The data indicate the presence of a significant population of IL-2R positive T cells in the spleens of GF-CD and conventional mice, but its minor presence in the lymph nodes.

TABLE 1. Flow cytometry analysis of IL-2R expression on spleen and lymph node cells from GF-CD and conventional BALB/c mice

Organ	$\alpha\beta$[1]	$\alpha\beta$IL-2R	IL-2R/$\alpha\beta$[2]	CD4	CD4IL-2R	IL-2R/CD4[3]
Spleen cells						
GF-CD	66.3	8.4	11.2	46.8	4.8	9.4
Conventional	61.4	3.7	5.6	47.9	2.6	5.2
Lymph node cells						
GF-CD	69.8	0.8	1.1	49.3	0.5	1.0
Conventional	71.3	0.6	0.8	52.1	0.4	0.8

1. Percentage of cells positive for TcR-$\alpha\beta$.
2. Percentage of cells in population of TcR-$\alpha\beta$ positive cells carrying the IL-2R.
3. Percentage of cells in population of CD4 positive cells carrying the IL-2R.

Limiting dilution analysis of high affinity IL-2R expression

The antibody to the IL-2R used for FACS analysis cannot distinguish the low

affinity from the functional high affinity IL-2R. In order to draw a more definite conclusion on the presence of activated T cells in the spleens of GF-CD mice, a limiting dilution analysis of purified T cells on IL-2 was performed. The data shown in Fig. 4 yield comparable frequencies of IL-2 responsive cells in both GF-CD and conventional spleens. These findings indicate the presence of activated T cells in mice that have not encountered exogenous antigenic stimulation.

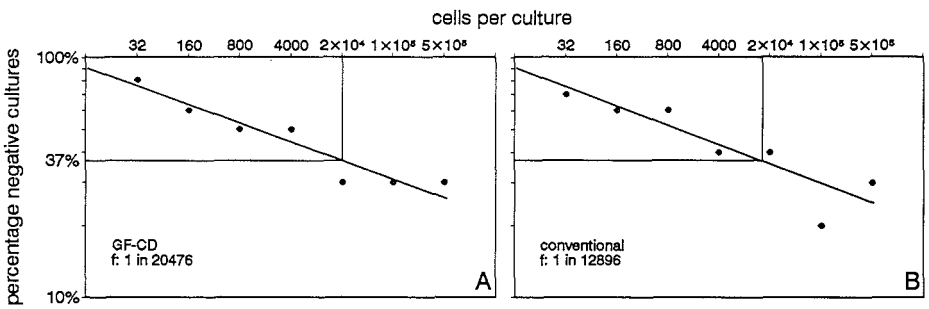

Figure 4. Limiting dilution analysis of functional IL-2R expression on spleen cells from **A**: GF-CD and **B**: conventional BALB/c mice.

DISCUSSION

The results of this investigation establish the presence of activated T cells in germfree mice fed a chemically defined ultrafiltered low molecular diet, which are considered to be antigen-free. One explanation for this background activity within the immune system could be, that it should be prepared at all times to defend the host against the exogenous threat of a variety of infectious agents. This concept is challenging, because it presumes that the immune system, which is an excellent learning-machine, even without any previous encounters with exogenous antigen, would know of such threats. It can of course be argued that evolutionary shaping of the immune system in hosts that have received multitudes of exogenous stimuli, has caused a genetic predisposition for this background activity. Ontogenetic and physiological studies, however, have shown that a variety of other cells, e.g. nerve cells, muscle cells and cells secreting various hormones, do not develop their specialized functions when they do not receive appropriate stimuli, and that they gradually lose these functions when these stimuli are stopped. The immune system could of course be an exception to this rule, but we favor another explanation for its background activity. The observed autonomous function in GF-CD mice could also reflect, that host-defense is only one of the functions of the immune system (12,13).

The presence of activated T cells in mice displaying only marginal levels of Ig of switched isotypes indicates the important role of the selectional processes within the thymus. Our results could suggest that thymically educated T cells display a low extent of autoreactivity that allows them to react to self-antigens, but not to secrete certain lymphokines after these encounters. From this point of view thymic education could be considered as a process of fine-tuning. This fine-tuning may be exemplified by the demonstration that T cell hybridomas only need 300 complexes of MHC molecules and specific antigenic peptides at the surface of antigen presenting cells to be stimulated (43). Combining the concept of thymic fine-tuning, the assumption that T cells have no *a priori* knowledge of the origin of the peptides that they recognize, and the demonstration of the ability of CD8-positive T cells to specifically lyse target-cells, it could be speculated that the immune system is a self-referential system very capable of providing host defense. From this point of view thymic education would learn T cells to respond to cells displaying sets of peptides quantitatively or qualitatively different from the ones encountered during positive selection in the thymus. These responses could result in the production of factors like IL-2, which can activate cytotoxic T cells, or factors like IL-4, which can activate B cells. According to this concept, which in this respect is similar to a hypothesis on alloreactivity (44), any cause of an altered set of peptides presented on MHC molecules, be it infection, transformation or any other cause of cellular dysfunction, could activate educated T cells to induce appropriate responses. The concept of the immune system as a self-referential system is speculative, but can be of great importance for our understanding of the various mechanisms that may cause autoimmune diseases.

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REFERENCES

1. Ehrlich, P. and Morgenroth, J. (1957) Reprinted in The collected papers of Paul Ehrlich, eds. Himmelweit, F., Marquardt, M. and Dale, H.D. (Pergamom, London), Vol. 2, p. 205.
2. Blackman, M., Kappler, J. and Marrack, P. (1990) Science 248: 1335.
3. Kappler, J.W., Roehm, M. and Marrack, P. (1987) Cell 49: 273.
4. Jenkins, M.K., Pardoll, D.M., Mitzuguchi, J., Chused, T.M. and Schwartz, R.H. (1987) Proc. Natl. Acad. Sci. USA 84: 5409.
5. Miller, R.D. and Calkins, C.E. (1988) J. Immunol. 140: 3779.

6. Sinha, A.A., Lopez, M.T. and McDevitt, H.O. (1990) Science 248: 1380.
7. Smith, J.B. and Pasternak, R.D. (1978) J. Immunol. 121: 1889.
8. Battisto, J.R. and Ponzio, N.M. (1981) Prog. Allergy 28: 169.
9. Glimcher, L.H. and Shevach, E.M. (1982) J. Exp. Med. 156: 640.
10. Jerne, N.K. (1974) Ann. Immunol. (Inst. Pasteur) 125: 373.
11. Vaz, N., Martinez-A, C. and Coutinho, A. (1984) in Idiotypy in Biology and Medicine, eds. Köhler, H., Cazenave, P.-A., Urbain, J. (Academic, New York), 142: 43.
12. Grabar, P. (1984) Immunol. Today 4: 337.
13. Coutinho, A., Forni, L., Holmberg, D., Ivars, F. and Vaz, N. (1984) Immunol. Rev. 79: 151.
14. Benner, R., van Oudenaren, A., Björklund, M., Ivars, F. and Holmberg, D. (1982) Immunol. Today 3: 243.
15. Pereira, P., Larsson, E.-L., Forni, L., Bandeira, A. and Coutinho, A. (1985) Proc. Natl. Acad. Sci. USA 82: 7691.
16. Hooijkaas, H., Benner, R., Pleasants, J.R. and Wostmann, B.S. (1984) Eur. J. Immunol. 14: 1127.
17. Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R. and Benner, R. (1988) Cell. Immunol. 112: 371.
18. Bos, N.A. and Meeuwsen, C.G. (1989) Eur. J. Immunol. 19: 1811.
19. Bos, N.A., Meeuwsen, C.G., van Wijngaarden, P. and Benner, R. (1989) Eur. J. Immunol. 19: 1817.
20. Pereira, P., Forni, L., Larsson, E.-L., Cooper, M., Heusser, C. and Coutinho, A. (1986) Eur. J. Immunol. 16: 685.
21. Vos, Q., Jones, L.A. and Kruisbeek, A.M., Chapter 4 of this thesis.
22. Pleasants, J.R., Johnson, M. and Wostmann, B.S. (1986) J. Nutr. 116: 1949.
23. Kappler, J.W., Skidmore, B., White, J. and Marrack, P. (1981) J. Exp. Med. 153: 1198.
24. Ozato, K., Mayer, N. and Sachs, D.H. (1980) J. Immunol. 124: 533.
25. Leo, O., Foo, M., Sachs, D.H., Samelson, L.E. and Bluestone, J.A. (1987) Proc. Natl. Acad. Sci. USA 84: 1374.
26. Hutchings, P.R., Cambridge, G., Tite, J.P., Meager, T. and Cooke, A. (1989) J. Immunol. Methods 120: 1.
27. Sedgwick, J.D. and Holt, P.G. (1983) J. Immunol. Methods 57: 301.
28. Vos, Q., Claassen, E. and Benner, R. (1990) J. Immunol. Methods 126: 89.
29. Kruisbeek, A.M., Mond, J.J., Fowlkes, B.J., Carmen, J.A., Bridges, S. and Longo, D.L. (1985) J. Exp. Med. 161: 1029.
30. Ceredig, R., Lowenthal, J.W., Nabholz, M. and MacDonald, H.R. (1985) Nature 314: 98.
31. Dialynas, D.P., Quan, Z.S., Wall, K.A., Pierres, A., Quintans, J., Loken, M.R., Pierres, M. and Fitch, F.W. (1983) J. Immunol. 131: 2445.
32. Kubo, R.T., Born, W., Kappler, J.W., Marrack, P. and Pigeon, M. (1989) J. Immunol. 142: 2736.
33. Hooijkaas, H., van der Linde-Preesman, A.A., Bitter, W.M., Benner, R., Pleasants, J.R. and Wostmann, B.S. (1985) J. Immunol. 134: 2223.
34. Coffman, R.L., Seymour, B.W.P., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Schrader, B., Cherwinsky, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W. and Mosmann, T.R. (1988) Immunol. Rev. 102: 5.
35. MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M. and Hengartner, H. (1988) Nature 332: 40.
36. Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. (1988) Nature 33: 742.

37. Kisielow, P., Teh., H.S., Blüthmann, H. and von Boehmer, H. (1988) Nature 335: 730.
38. Zuñiga-Pflücker, J.C., Longo, D.L. and Kruisbeek, A.M. (1989) Nature 338: 76.
39. Blackman, M.A., Marrack, P. and Kappler, J. (1989) Science 244: 214.
40. Nikolić-Zugić, J. and Bevan, M. (1990) Nature 344: 65.
41. Malek, T.R., Robb, R.J. and Shevach, E.M. (1983) Proc. Natl. Acad. Sci. USA 80: 5694.
42. Greene, W.C. and Leonard, W.J. (1986) Annu. Rev. Immunol. 4: 69.
43. Harding, C.V. and Unanue, E.R. (1990) Nature 346: 574.
44. Matzinger, P. and Bevan, M.J. (1977) Cell. Immunol. 29: 1.

CHAPTER 6

IN VIVO TREATMENT WITH MONOCLONAL ANTI-I-A ANTIBODIES CAUSES A RAPID DISAPPEARANCE OF SPLENIC T CELLS AND BACKGROUND IMMUNOGLOBULIN SECRETING CELLS

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SUMMARY

We have studied the effect of *in vivo* treatment with anti-I-A antibody on the background immunoglobulin secreting cells (IgSC) in the murine spleen. For this purpose groups of C57BL/6 mice received two intraperitoneal injections of monoclonal anti-I-A^b or control anti-I-A^k antibody of the IgG2a isotype. The treatment with anti-I-A^b antibody resulted in a rapid decrease of the total number of spleen cells, the number of splenic T cells and the frequency of background IgSC, which was most predominant for the non-IgM isotypes. These data suggest that interactions between MHC class II restricted T cells and antigen-presenting cells carrying these molecules are involved in the development of background IgSC, and in peripheral T cell renewal. The rapid disappearance and reappearance of both cell types after anti-I-A treatment indicate a high turnover of these cells in the murine spleen.

INTRODUCTION

CD4-positive T cells require concomitant recognition of antigen and MHC class II molecules to respond to antigenic stimulation by lymphokine production and proliferation [1-4]. Antibodies against MHC class II molecules have been used to study the functional role of surface I-A molecules in *in vitro* assays [5-12]. These studies

have demonstrated that the inhibition of T cell function by anti-I-A antibodies *in vitro* is not due to a direct effect of the antibodies on the T cell, but to an interference in the recognition of I-A determinants [5-7].

Studies using *in vivo* administration of anti-I-A antibodies have shown the inhibitory effect of this treatment on the immune responses against a variety of antigens [13,17]. Moreover, *in vivo* treatment with anti-I-A antibodies has been shown to prevent graft-rejection [13] and graft-vs-host disease [14], as well as to suppress experimental myasthenia gravis [18], experimental encephalitis [19] and autoantibody production in (NZB x NZW)F1 mice [20]. Investigations of the mechanisms responsible for the loss of antigen presenting function of spleen cells treated *in vivo* with anti-I-A antibodies have shown a down-regulation of surface I-A and I-E expression, which was dependent of the dose of antibody administered [21,22].

Unmanipulated mice display background activity within the immune system which has been demonstrated for B [23] as well as T cells [24]. A comparison of the background IgSC from conventional mice and mice living in complete absence of exogenous antigenic stimulation [25,26] has indicated the role of exogenous antigens in the activation of T cells to secrete lymphokines inducing isotype-switching [27] in the B cells of conventional mice.

In order to study the importance of T cell - I-A interactions in the development of splenic background IgSC, we have treated mice *in vivo* with anti-I-A antibody. This treatment resulted in a rapid decrease of background IgSC, which was most predominant for cells secreting the non-IgM isotypes, and in a rapid recovery thereafter. This finding indicated the role of T cell - MHC class II interactions in the development of background IgSC. The anti-I-A treatment also caused a decrease in the total number of spleen cells and the number of splenic T cells, indicating the importance of MHC class II restricted interactions in peripheral T cell renewal, as well.

MATERIALS AND METHODS

Mice

C57BL/6 mice, 8 to 12 weeks of age, were obtained from the National Cancer Institute (Frederick, MD).

In vivo anti-I-A treatment

Anti-I-A^b, Y3P [28], and control anti-I-A^k, 10.3.6 [29], were purified from nude mice ascites by ammonium sulphate precipitation and Sephadex column size

separation. Concentrated antibodies were dialyzed against PBS, filter sterilized, and the concentration of the antibodies was determined by spectrophotometric absorbance. Mice were randomly divided in two groups, one receiving anti-I-A^b, the other the control anti-I-A^k. Mice received two intraperitoneal injections of antibody: 0.5 mg at day 0, and 0.2 mg at day 1.

Enumeration of splenic T cells

Splenic T cells were isolated by treatment of splenocytes with anti-I-A^b (Y3P) and complement followed by density gradient separation (Lympholyte-M; Cedar Lane, Ontario, Canada). Cells were counted using a hemocytometer.

Enumeration of background IgSC

IgSC of various isotypes were detected by the spot ELISA [30] with some modifications described previously [31].

RESULTS AND DISCUSSION

Effect of *in vivo* anti-I-A treatment on the total number of spleen cells

In order to study the effect of *in vivo* treatment with anti-I-A antibody, groups of C57BL/6 mice, chosen for their lack of I-E expression, were treated with two injections, 0.5 mg on day 0 and 0.2 mg on day 1, of purified anti-I-A^b or control anti-I-A^k antibody, both of murine origin and IgG2a isotype. The dosage scheme was chosen since previous work [22] indicated that this treatment would result in a reappearance of I-A surface expression at day 2 after the first injection. On the first day of treatment already a significant reduction of the total number of spleen cells was found (Fig. 1). The data show a maximal decrease of 60% at day 2 for anti-I-A^b treatment, whereas no reduction was observed after control treatment. The data also indicate a rapid recovery at day 3, one day after the previously indicated moment of I-A reexpression. Both anti-I-A^b and, more predominantly, control treatment caused an increase in the total numbers of spleen cells at day 4, which was also demonstrated for the number of splenic T cells and background IgSC. It could be speculated that *in vivo* administration of a high dose of antibodies, likely to contain some mitogenic impurities next to their idiotypic determinants, can induce such a response. Previously it has been shown that immunisation of mice with sheep red blood cells could induce a significant increase of background IgSC at day 4 after injection [32]. Comparison of

time and order of magnitude could indicate that the increase observed in the present experiments was caused by an aspecific response to the injected antibodies.

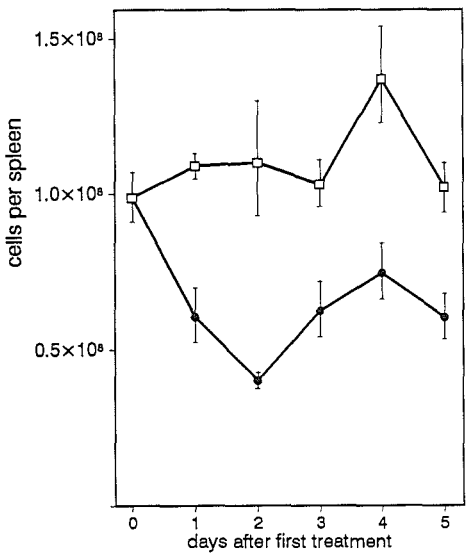

Figure 1. Effect of *in vivo* treatment with anti-I-A antibodies on the total numbers of spleen cells. ● : anti-I-A^b treatment; □ : control anti-I-A^k treatment. Results are shown as the geometric mean ± S.E.M. of 5 individually tested mice.

Effect of *in vivo* anti-I-A treatment on the number of I-A negative spleen cells

The data in Fig. 1 show a 60% reduction of spleen cells at day 2 after the first anti-I-A^b treatment. Assuming that about 50% of all spleen cells are B cells and that the administered dose of antibody could not have removed all I-A^b positive cells, it could be concluded that B cells were not the only population which was reduced by the treatment. FACS analysis was performed to test this hypothesis, but *in vivo* treatment with anti-I-A antibody increased the aspecific backgrounds of both groups to such extent, that no reliable analysis could be done. In order to have an indirect way to quantitate a group of cells different from background IgSC, spleen cells of each group were pooled and treated with anti-I-A^b and complement to determine the pool size of splenic I-A-negative cells, presumably largely T cells. Fig. 2 shows the effects of both treatments on this T cell enriched fraction and indicates a rapid decrease of splenic T cells after anti-I-A^b treatment. Since the anti-I-A^k antibody did not induce a comparable reduction, the effects of anti-I-A^b treatment cannot be explained by a removal of cells binding the administered antibodies via Fc-receptors. The aspecific response at day 4 was already discussed above. Based on reports on the daily thymic T cell output [33], the rapid disappearance and reappearance cannot be explained by demonstrated

effects [34] of anti-I-A treatment on the thymus. Our data therefore indicate the importance of MHC class II interactions for the maintenance of the peripheral T cell pool and support reports on the high turnover rate of peripheral T cells [35,36].

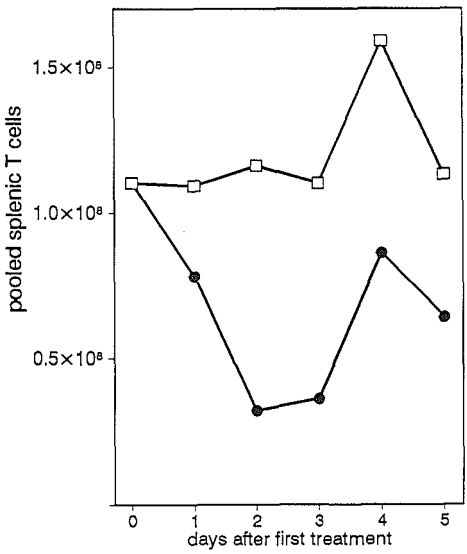

Figure 2. Effect of *in vivo* treatment with anti-I-A antibodies on the number of splenic I-A negative (T) cells. ● : anti-I-A^b treatment; □ : control anti-I-A^k treatment. Results are the numbers resulting from a pool of I-A negative spleen cells from 5 mice.

Effect of *in vivo* anti-I-A treatment on splenic background IgSC of various isotypes

Since treatment with control anti-I-A^k antibody caused an aspecific response, we have used the following protocol for data analysis. Each day the data of 5 individual mice per treatment were collected and their geometric mean was calculated. In order to compensate for aspecific effects the mean of the anti-I-A^b group was divided by the mean of the control group. Fig. 3 shows the resulting percentages ± S.E.M. for the isotypes assayed. The data indicate a rapid reduction of IgSC of all isotypes, with the smallest decrease and fastest recovery for IgM secreting cells and a reduction of 60% for IgG1-SC at day 3. These data support a report on the short half-life and high renewal rate of background IgSC in the spleen [37].

The observed reduction of IgSC can be explained by three mechanisms: (a) cytotoxicity of the administered antibody; (b) interference with T cell independent B cell maturation; and (c) activation and blocking of T cell influence on these processes. The difference in kinetics of IgM- and IgG1-SC indicate that direct cytotoxicity cannot solely be responsible for the effects of anti-I-A^b treatment. Direct effects of anti-MHC class II

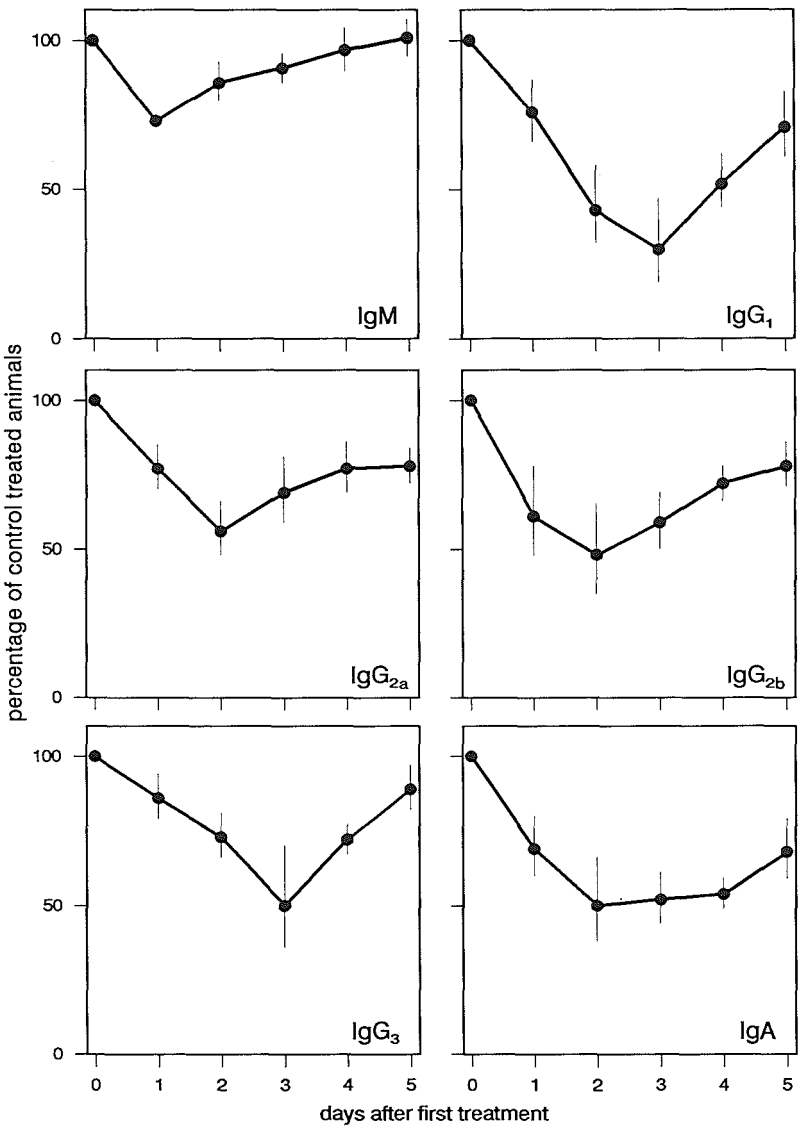

Figure 3. Effect of *in vivo* treatment with anti-I-A antibodies on the numbers of IgSC of various isotypes in the spleen. Results are shown as the percentage of cells compared with a negative control group. Results are shown as the geometric mean ± S.E.M. of 5 individually tested mice.

antibodies on the activation of human B cells have been shown [38,39] and can be a cause of the observed effects, especially the reduction of the pool of immature B cells indicated by Figs. 1 and 2. It can, however, not account for the differences between the various isotypes. With regard of the observed reduction of splenic B cells, the data shown in Fig. 3 can most likely be explained by interference with T cell dependent B cell differentiation and activation. Bearing in mind that the frequency of splenic IgM-SC is approximately 25 times higher than of IgG-SC of various isotypes, we propose the following hypothesis on the mechanism causing the effects of *in vivo* anti-I-A treatment shown in this communication.

Injection of anti-I-A antibody causes a limited reduction of all I-A positive cells by direct cytotoxicity, which will be most predominant directly after injection, and by a direct effect on B cell maturation involving a blocking and down-regulation of surface I-A expression. This down-regulation interferes with T cell induced B cell switching from IgM to the other isotypes causing an extra reduction of these. The blocking also interferes with the signals that T cells need for their peripheral renewal, causing their decrease and a further reduction of the numbers of cells secreting "switched" isotypes. As soon as anti-I-A levels drop, MHC class II molecules start to reappear and a return to the normal situation occurs.

ACKNOWLEDGMENTS

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REFERENCES

1. Thomas, E.W., Yamashita, U. and Shevach, E.M., Immunol. Rev. 1977. 35:116.
2. Schwartz, R.H., Yano, A. and Paul, W.E., Immunol. Rev. 1978. 40:153.
3. Möller, G. ed., Immunol. Rev. 1978. 42
4. Berzofsky, J.A., in Goldberger, R.E. (Ed.), Biological Regulation and Development, Plenum Press, New York 1980. Vol. II, p. 467.
5. Hodes, R.J., Hathcock, K.S. and Singer, A., J. Exp. Med. 1980. 152:1779.
6. Longo, D.L. and Schwartz, R.H., Proc. Natl. Acad. Sci. USA 1981. 78:514.
7. Nepom, J.T., Benacerraf, B. and Germain, R.N., J. Immunol. 1981. 127:31.
8. Niederhuber, J.E., Immunol. Rev. 1978. 40:28.
9. Schwartz, R.H., David, C.S., Sachs, D.H. and Paul, W.E., J. Immunol. 1976. 117:531.
10. Marrack, P. and Kappler, J.W., Immunogenetics 1977. 4:541.
11. Shevach, E.M., Green, I. and Paul, W.E., J. Exp. Med. 1974. 139:679.
12. Berzofsky, J.A. and Richman, L.K., J. Immunol. 1981. 126:1898.
13. Davis, D.A.L. and Staines, N.A., Transplant. Rev. 1976. 30:18.

14. Prud'homme, G.J., Sohn, U. and Delovitch, T.L., J. Exp. Med. 1979. 149:137.
15. Sprent, J., J. Exp. Med. 1980. 152:996.
16. Rosenbaum, J.T., Adelman, N.E. and McDevitt, H.O., J. Exp. Med. 1981. 154:1694.
17. Perry, L.L. and Greene, M.I., J. Exp. Med. 1982. 156:480.
18. Waldor, M.K., Sriram, S., McDevitt, H.O. and Steinman, L., Proc. Natl. Acad. Sci. USA 1983. 80:2713.
19. Sriram, S. and Steinman, L., J. Exp. Med. 1983. 158:1362.
20. Adelman, N.E., Watling, D.L. and McDevitt, H.O., J. Exp. Med. 1983. 158:1350.
21. Fultz, M., Finkelman, F.D. and Mond, J.J., J. Immunol. 1984. 133:91.
22. Kruisbeek, A.M., Titus, J.A., Stephany, D.A., Gause, B.L. and Longo, D.L., J. Immunol. 1985. 134:3605.
23. Benner, R., van Oudenaren, A., Björklund, M., Ivars, F. and Holmberg, D., Immunol. Today 1982. 3:243.
24. Pereira, P., Larsson, E.-L., Forni, L., Bandeira, A. and Coutinho, A., Proc. Natl. Acad. Sci. USA 1985. 82:7691.
25. Hooijkaas, H., Benner, R., Pleasants, J.R. and Wostmann, B.S., Eur. J. Immunol. 1984. 14:1127.
26. Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R. and Benner, R., Cell. Immunol. 1988. 112:371.
27. Coffman, R.L., Seymour, B.W.P., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Schrader, B., Cherwinsky, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W. and Mosmann, T.R., Immunol. Rev. 1988. 102:5.
28. Janeway, C.A., Jr., Conrad, P.J., Lerner, E.A., Babich, J., Wettstein, P. and Murphy, D.B., J. Immunol. 1984. 132:662.
29. Oi, V.T., Jones, P.P., Goding, J., Herzenberg, L.A. and Herzenberg, L.A., Curr. Top. Microbiol. Immunol. 1978. 81:115.
30. Sedgwick, J.D. and Holt, P.G., J. Immunol. Methods 1983. 57:301.
31. Vos, Q., Claassen, E. and Benner, R., J. Immunol. Methods 1990. 126:89.
32. Benner, R., Rijnbeek, A.-M., van Oudenaren, A. and Coutinho, A., Adv. Exp. Med. Biol. 1982. 149:703.
33. Scollay, R., Butcher, E. and Weissman, I., Eur. J. Immunol. 1980. 10:210.
34. Kruisbeek, A.M., Mond, J.J., Fowlkes, B.J., Carmen, J.A., Bridges, S. and Longo, D.L., J. Exp. Med. 1985. 161:1029.
35. Rocha, B., Freitas, A.A. and Coutinho, A.A., J. Immunol. 1983. 131:2158.
36. Rocha, B., Larsson, E.-L. and Freitas, A.A., Scand. J. Immunol. 1984. 19:315.
37. Lévy, M., Vieira, P., Coutinho, A. and Freitas, A., Eur. J. Immunol. 1987. 17:849.
38. Holte, H., Blomhoff, H.K., Beiske, K., Funderud, S., Torjesen, P., Gaudernack, G., Stokke, T. and Smeland, E.B., Eur. J. Immunol. 1989. 19:1221.
39. Hamano, T., Iwasaki, T., Yamasaki, T., Murata, Y., Kakashita, E. and Nagai, K. J. Immunol. 1990. 144:811.

CHAPTER 7

GENERAL DISCUSSION

7.1.1 The results from Chapter 3

The results shown in Chapter 3 demonstrate that the usage of a technique employing coated capture antibodies and enzyme-labeled insulin can greatly improve the sensitivity of the conventional spot-ELISA for the detection of cells secreting antibodies of the IgG isotypes. Moreover an improved protocol for the detection of immunoglobulin-secreting cells (IgSC) was described, which was used in some of the experiments described in Chapters 4 to 6.

7.1.2 The results from Chapter 4

The results shown in Chapter 4 demonstrate that functional T cells can develop in mice that have not received exogenous antigenic stimulation. Moreover, analysis of the expression of T cell receptor Vβ chains on mature thymocytes and peripheral T cells from germfree mice fed a chemically defined diet (GF-CD) indicated no significant differences with the expression in conventional mice. These results have a number of important implications.

Firstly they show the high degree of autonomy in T cell development. This autonomy indicates that self-antigens are a sufficient source of peptides for the development of thymocytes by both positive and negative selection and the maintenance of peripheral T cells.

Secondly they indicate that the influence of exogenous antigens on these processes is of a limited nature only.

Thirdly they challenge current concepts on autoreactivity of T cells as they support evidence on the role of self-peptides in the positive selection of thymocytes and indicate that self-recognition can result in the maturation to mature T cells.

7.1.3 The results from Chapter 5

The results from the experiments described in Chapter 5 give a further illustration of the functional properties of GF-CD T cells and indicate the presence of activated cells within this population.

The response of GF-CD T cells to allogeneic stimulator cells demonstrates their ability to be triggered by stimuli involving T cell receptor (TcR) mediated recognition of MHC molecules and associated peptides. Another not eye-catching but yet important result, which will be discussed further on, is the lack of response of GF-CD T cells to cells from syngeneic conventional mice.

The results from the *in vitro* stimulation experiments indicate that GF-CD T cells are able to respond to TcR crosslinking by lymphokine secretion and that GF-CD B cells can be induced to isotype switching. These findings demonstrate that the severely reduced numbers of IgSC of the non-IgM isotypes detected in GF-CD mice are not caused by functional defects in their T and/or B cells.

The results from the flow cytometry analysis of IL-2 receptor expression and from the limiting dilution analysis of GF-CD T cells on IL-2 indicate the presence of activated T cells in mice that have not received exogenous antigenic stimulation. Together with the earlier results from studies on the B cell compartment of GF-CD mice these findings demonstrate functional activity within the immune system of these mice. This activity is due to an internal activation within the immune system since the GF-CD mice do not need, and actually do not mount, a host-defense against invasive pathogenic micro-organisms.

7.1.4 The results from Chapter 6

The results shown in Chapter 6 demonstrate the importance of cell interactions involving the recognition of MHC molecules and associated peptides for the internal activation within the immune system. The rapid disappearance of IgSC of the non-IgM isotypes after *in vivo* treatment with anti-MHC class II antibodies indicates the importance of helper T cells and their isotype-switch inducing lymphokines on the development of these cells. The small decrease in cells secreting IgM indicate the limited influence of T cells on their differentiation.

This stands in clear contrast to the dependence of IgSC producing other isotypes on T cell - MHC class II interaction. The decrease in the number of peripheral T cells after treatment illustrates the necessity of the interaction between helper T cells and antigen presenting cells for the maintenance of the T cell pool. Although the results from Chapter 6 allow no direct conclusion, it may be speculated on the basis

of reports on the peripheral renewal of T cells, that the observed decrease is the result from a blockade of the stimuli inducing the peripheral clonal expansion of T cells. The kinetics of the phenomena observed give further proof to previous observations on the short half life and the high renewal rate of the majority of cells within the pool of peripheral lymphocytes.

7.2 Self, non-self and the immune system

Studies on the properties of the receptors expressed by mature T cells have shown that 1 in every 10,000 T cells reacts with a certain peptide presented by a syngeneic cell (1). Such studies have also demonstrated that the presence of 300 complexes of a specific peptide with MHC on the surface of an antigen-presenting cell (i.e. an 0.1% occupation of all MHC molecules) is sufficient to trigger T cells (2). These findings suggest that T cells resulting from thymic education carry receptors with a high degree of antigenic specificity, and display a high sensitivity in their specific recognition.

Due to the absence of exogenous antigenic stimulation, in GF-CD mice both positive (3) and negative selection (4) of thymocytes can only be induced by the recognition of self-peptides presented in the context of self-MHC molecules.

The identical patterns of clonal deletion observed in GF-CD and conventional mice support on the one hand current ideas on the importance of negative selection for the removal of thymocytes displaying self-reactivity, since the GF-CD mice have self-antigens as the only source of MHC associated peptides.

The demonstration of mature thymocytes and of functional T cells in GF-CD mice, on the other hand, support a report indicating the importance of the recognition of MHC associated self-peptides in the positive selection of thymocytes (5).

This seemingly contradictory role of self-recognition in two processes of opposed nature can be explained by the interpretation of negative and positive selection as two mechanisms setting the upper and lower limits in a process of fine-tuning. This fine-tuning would result in a population of mature thymocytes that display a well-defined low extent of self-recognition. Taking into account the observations mentioned in the beginning of this section, such a low extent of self-reactivity could be based on the recognition of a particular self-peptide which is presented by MHC molecules on the surface of thymic antigen-presenting cells like epithelial cells, macrophages or dendritic cells in numbers lower than about 300 per cell. From this point of view the autoreactivity which T cells have been demonstrated to display under *in vitro* culture conditions (6-8) can be explained by the increased presentation of self-peptides that these conditions allow. The reactivity to non-self peptides could be explained by

cross-reactivity of T cell receptors (9).

Besides this new concept on T cells and autoreactivity the results presented in Chapters 5 and 6 could support some ideas on its consequences.

The results of the allogeneic stimulation assays presented in Chapter 5 indicate that GF-CD T cells do not respond *in vitro* to cells from syngeneic conventional mice. Since the flow cytometry analysis of GF-CD T cells indicated the same repertoire of TcR Vβ chains as conventional mice, this lack of response could mean that the repertoire of peptides presented by splenic antigen presenting cells from conventional mice is similar to that of corresponding cells from GF-CD mice, and consists mainly of self-peptides.

Based on the findings of Chapter 6, which indicated that an interference in the recognition of MHC molecules resulted in a rapid decrease of the peripheral T cell pool, it could be speculated that the maintenance of the pool of peripheral T cells, which is highly dependent of cell division (10), is induced by the recognition of MHC-associated self-peptides (11). This speculation would indicate a dramatic contrast between the way T cells respond to syngeneic stimuli under *in vitro* and *in vivo* conditions.

This speculation and its theoretical backgrounds may seem highly improbable, but on the other hand the data from Chapters 5 and 6 support a report on the differences between T cell proliferation under *in vitro* and *in vivo* conditions (12). Based on these ideas thymic education would lead to a repertoire of T cells whose low extent of self-reactivity allows them on the one hand to divide upon interaction with self-peptides, but on the other hand not to initiate an autoimmune response.

Any perturbation of the set of peptides presented by the peripheral antigen-presenting cells could activate the immune system to humoral and or cellular responses which allow the establishment of a new state of homeostasis. These perturbations could be induced by exogenous antigens leading the immune system to function as a host defense system, or could be induced by increased amounts of self antigens or by "neo-self" antigens caused by biochemical malfunctions, mutations or transformations.

From this point of view the immune system could be defined as the molecular equivalent of the nervous system with a task as a self-referential system (13), monitoring the molecular homeostasis of a large variety of specialized differentiated cells. In contrast to current ideas, self-recognition and self-assessment would be of great importance for this system and could be the stimuli responsible for the completeness and functional status of the immune system.

References

1. Marrack, P. and Kappler, J. 1988. Immunol. Today 9: 308.
2. Harding, C.V. and Unanue, E.R. 1990. Nature 346: 574.
3. Teh, H.S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Blüthmann, H. and von Boehmer, H. 1988. Nature 335: 229.
4. Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. 1988. Nature 333: 742.
5. Nikolić-Zugić, J. and Bevan, M. 1990. Nature 344: 65.
6. Smith, J.B. and Pasternak, R.D. 1978. J. Immunol. 121: 1889.
7. Battisto, J.R. and Ponzio, N.M. 1981. Prog. Allergy 28: 169.
8. Glimcher, L.H. and Shevach, E.M. 1982. J. Exp. Med. 156: 640.
9. Claverie, J.M. and Kourilsky, P. 1986. Ann. Immunol. (Inst. Pasteur) 137D3: 425.
10. Rocha, B., Freitas, A.A. and Coutinho, A.A. 1983. J. Immunol. 131: 2158.
11. Rocha, B. and von Boehmer, H. Personal communication.
12. Rocha, B., Lembezat, M.-P., Freitas, A. and Bandeira, A. 1989. Eur. J. Immunol. 19: 1137.
13. Coutinho, A., Forni, L., Holmberg, D., Ivars, F. and Vaz, N. 1984. Immunol. Rev. 79: 151.

SUMMARY

This thesis describes three areas of immunological research:

- Firstly the development of an immunological technique which allows an improved detection of cells secreting specific antibodies. The results of this study demonstrated that the use of a protocol employing coated capture antibodies and enzyme-labeled antigen in stead of the sandwich-method employing antigen coating and enzyme-labeled detector antibodies could considerably improve the detection of cells secreting antibodies of the IgG isotypes.
- Secondly an investigation of the immune system of germfree mice, fed an ultrafiltered chemically defined low-molecular diet. Such mice are considered to be completely free of exogenous antigens. Previous studies have shown that such mice have similar numbers of B cells and background IgM secreting cells as conventional mice, but are highly deficient in background IgG and IgA production. The results of our study demonstrated that such mice had a normal repertoire of functional T cells, that could be induced to lymphokine secretion, and that the absence of background immunoglobulin secreting cells of the non-IgM isotypes was not caused by defects in their B or T cells. This data indicates that the immune system has an autonomous activity, which is independent of exogenous antigenic stimulation.
- Thirdly a study on the effects of the manipulation of the immune system with antibodies against MHC class II molecules. The results of this study demonstrated that *in vivo* treatment with antibodies directed against the MHC class II molecules caused a rapid decrease in the number of background immunoglobulin secreting cells and T cells in the spleen. This indicates that the generation of background immunoglobulin secreting cells and the maintenance of the T cell compartment are dependent on cognate interactions within the immune system involving MHC class II.

The combined results from these studies indicate that self-recognition is of great importance for the autonomous activity of the immune system.

By defining the immune system as a host-defense system, it has been postulated that the cells involved in this system should react to foreign antigens, but ignore self-molecules. Based on the observation of self-recognition within the system we conclude that host-defense is one among the tasks of the immune system, which could be considered as the molecular equivalent of the nervous system.

SAMENVATTING

Dit proefschrift beschrijft drie terreinen van immunologisch onderzoek:

- Ten eerste de ontwikkeling van een immunologische techniek, die een verbeterde detectie van antilichaam-secernerende cellen mogelijk maakt. Deze techniek maakt gebruik van geïmmobiliseerde antilichamen en enzym-geconjugeerd antigeen in plaats van de toepassing van de sandwich-methode, die gebaseerd is op de immobilisatie van antigeen en detectie met enzym-geconjugeerde tweede staps antilichamen. De resultaten van dit onderzoek tonen aan dat deze nieuwe techniek de detectie van IgG antilichaam secernerende cellen aanzienlijk kan verbeteren.
- Ten tweede een onderzoek van het immuunsysteem van niet-geïmmuniseerde kiemvrije muizen, die gevoed werden met een ultragefiltreerd dieet bestaande uit laagmoleculaire voedingsstoffen. Eerdere studies hebben aangetoond dat zulke muizen aantallen B-cellen en IgM secernerende cellen bezitten, die vergelijkbaar zijn met conventionele muizen, maar een grote deficiëntie in "achtergrond" IgG en IgA productie hebben. Deze muizen worden geheel vrij van exogene antigenen beschouwd. De resultaten van onze studie tonen aan, dat zulke muizen een normaal repertoire van functionele T-cellen hebben die tot lymfokine secretie kunnen worden gestimuleerd, en dat de afwezigheid van "achtergrond" immunoglobuline secernerende cellen van andere dan IgM isotype niet veroorzaakt wordt door defecten in hun B- of T-cellen. Dit toont aan dat het immuunsysteem een autonome activiteit bezit, die onafhankelijk is van exogene antigene stimulatie.
- Ten derde een onderzoek naar de gevolgen van manipulatie van het immuunsysteem met antilichamen tegen MHC klasse II moleculen. De resultaten van deze studie tonen aan dat *in vivo* behandeling met antilichamen gericht tegen MHC klasse II moleculen een snelle verlaging veroorzaakte van het aantal "achtergrond" immunoglobuline secernerende cellen en het aantal T-cellen in de milt. Dit duidt erop dat de vorming van "achtergrond" immunoglobuline secernerende cellen en de instandhouding van het T-cel compartiment afhankelijk zijn van "cognate" interacties binnen het immuunsysteem via MHC klasse II moleculen.

De gezamenlijke resultaten van deze studies tonen aan dat zelf-herkenning van groot belang is voor de autonome activiteit binnen het immuunsysteem.

Door het immuunsysteem als *afweersysteem* te definiëren, is gepostuleerd dat deze celpopulatie moest reageren op lichaamsvreemde antigenen, maar lichaamseigen antigenen moest negeren. Uitgaande van de waargenomen zelf-herkenning binnen het systeem concluderen wij dat de afweer tegen lichaamsvreemde antigenen slechts één van de taken van het immuunsysteem is, dat zou kunnen worden gezien als een moleculair equivalent van het zenuwstelsel.

LIST OF ABBREVIATIONS

AMP	: 2-amino-2-methyl-1-propanol
AP	: alkaline phosphatase
ASC	: antibody-secreting cell(s)
5-BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
BSA	: bovine serum albumin
CFA	: complete Freund's adjuvant
DMEM	: Dulbecco's modified Eagle's medium
ELISA	: enzyme-linked immunosorbent assay
FCS	: fetal calf serum
FITC	: fluorescein isothiocyanate
GA	: glutaraldehyde
GAM	: goat antiserum directed against mouse Ig
GAM-AP	: conjugate of GAM and AP
GF-CD	: germfree (mice), fed a chemically defined ultrafiltered low-molecular diet
HBSS	: Hank's balanced salt solution
HI	: human insulin
HI-AP	: conjugate of HI and AP
IFN-γ	: gamma interferon
Ig	: immunoglobulin(s)
IgSC	: immunoglobulin-secreting cell(s)
IL-2	: interleukin 2
IL-2R	: interleukin 2 receptor
LPS	: lipopolysaccharide
MAb	: monoclonal antibody
2-ME	: β-mercaptoethanol
MHC	: major histocompatibility complex
MLS	: minor lymphocyte stimulating antigen
PBS	: phosphate-buffered saline
PBT 0.1	: PBS containing 0.1% BSA and 0.1% Tween 20
PBT 1.0	: PBS containing 1.0% BSA and 0.1% Tween 20
PLN	: popliteal lymph nodes
PNP	: p-nitrophenyl phosphate
RAM	: rat MAb directed against mouse Ig
SPF	: specific pathogen free
sIg	: surface immunoglobulin(s)
TcR	: T cell receptor

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 29 maart 1961 te Rotterdam geboren, alwaar hij van 1965 tot 1973 de Rotterdamse School Vereniging en van 1973 tot 1980 het Gymnasium Erasmianum bezocht. Hierna studeerde hij van 1980 tot 1985 Pharmacie te Leiden en was van 1985 tot 1987 werkzaam op het Rikilt te Wageningen. Vanaf 1987 werkte hij op de afdeling Immunologie van de Erasmus Universiteit te Rotterdam, gedurende welke periode het in dit proefschrift beschreven onderzoek werd uitgevoerd. Gedurende deze periode werd een werkbezoek gebracht aan het laboratorium van Dr. A.M. Kruisbeek op het National Cancer Institute van de National Institutes of Health, Bethesda, USA.