

Dissection of Systems, Cell Populations and Molecules

R. Benner & N. A. Khan

Department of Immunology, Erasmus
MC – University Medical Center Rotterdam,
Rotterdam, The Netherlands

Received 8 December 2004; Accepted in revised
form 25 January 2005

Correspondence to: Dr R. Benner, PhD,
Department of Immunology, Erasmus MC, P.O.
Box 1738, 3000 DR Rotterdam, The
Netherlands. E-mail: r.benner@erasmusmc.nl

Abstract

This paper summarizes studies on antibody formation in the bone marrow and the suppressive effects of intravenous immunization with allogeneic blood cells on T-cell function in mice. The latter studies were extended by employing the limiting dilution culture system developed in Ivan Lefkovits' laboratory and implemented in collaboration with Lucien Aarden. Thereby, the functional data were complemented with frequencies of alloantigen-activated helper (Th) and suppressor T cells after intravenous alloimmunization. These results led the Rotterdam group to studies on the prevention of rejection of the foetal 'allograft'. Th cells are central in foetal allograft rejection and pregnancy success. Characteristic for human pregnancy is the production of the glycoprotein chorionic gonadotropin (hCG) hormone. The *in vivo* liberated peptide fragments originating from nicking of the sequence MTRVLQGVLPALPQ in the β -chain of hCG were considered for their immunoregulating capacity related to pregnancy success. These peptides – prepared synthetically – (MTR, MTRV, LQG, LQGV, VLPALP and others) indeed showed a remarkable spectrum of biological effects (e.g. modulation of angiogenesis, inhibition of septic shock syndrome, prevention of diabetes and reduction of ischaemia-reperfusion damage). The paper interprets and generalizes these findings and projects them into various research directions, especially towards the proteomics framework studies built up in Ivan Lefkovits' laboratory in the nineties. During the time period, when Ivan spent a mini-sabbatical in Rotterdam (months after closing down the BII) more detailed discussions were initiated. This paper is meant to keep the discussions between the involved research groups going on.

Introduction

Talking with Ivan Lefkovits about any subject, be it immunity, scientific institutions, the scientific community, the lay-society or society as a whole, is a discussion about systems, components and interaction between these components, their origin and possible further development. Notion, insights. How Niels Jerne (Nobel Prize 1984) would have viewed it when he, like Kierkegaard [1], turned matters upside down: the ideas and scientific publications of Niels Jerne as a portrait of the immune system and other systems [2]. The Erasmus University of Rotterdam often quotes [3] its namegiver for his sayings 'ad fontes' (to the sources) and 'suis oculis cernere' (to see/discriminate with one's own eyes). These sayings also characterize Ivan's attitude towards the ideas and scientific work of Niels Jerne. Erasmus, the great humanist, theologian, philosopher and writer was born in Rotterdam (probably in 1469) and died in Basel (1536). Having originated at the Erasmus

University of Rotterdam and arriving at the Basel Institute for Immunology (in 1978), the Rotterdam–Basel connection became clearer to me (RB) than ever before. Niels Jerne was raised and received his education in Rotterdam, and, being the famous director of 'The Institute', liked to speak Dutch with the Dutch in the Institute. Erasmus seemed to be everywhere in Basel: his grave in the cathedral, famous paintings made of him in the Kunstmuseum, a memorial plaque for him on the front of Haus zum Luft in the Bäumleingasse, and a square, a restaurant and an antiquariat are named after him. But, maybe most importantly in my feeling so welcome in Basel were Ivan Lefkovits and his crew in laboratory 12, including Lucien Aarden and, a frequent visitor, Annemarie Rijnbeek, both also from Holland. To me, Ivan appeared the real host of The Institute. If anything was not solved by the fantastic services led by Mr Bron, it was solved by Ivan or his kind technicians Anita Söderberg and Pat Young.

Dissection of systems: antibody formation in the bone marrow

Before coming to The Institute, most of my studies were on antibody formation in the bone marrow. Although studies, suggesting that the bone marrow can produce antibodies, had already been published by the end of the 19th century [4, 5], in the seventies of the 20th century textbooks still did not mention the bone marrow as a source of antibodies after infection or immunization. The well-known presence of plasma cells in the human bone marrow was generally considered as the consequence of the graveyard function of the organ, the recruitment of old and damaged haemopoietic and lymphoid cells to the marrow and their subsequent break down. This was probably due to the fact that till then the bone marrow of the most popular experimental animal in immunological research, the mouse, was found to have little or no antibody-forming cells after immunization and application of Niels Jerne's plaque-forming cell assay. However, these initial studies in mice were limited in one respect, and this appeared to be a crucial one: antibody formation in the bone marrow had been studied during primary responses only, and these studies were restricted to the first 1 or 2 weeks after immunization. Later after primary immunization, and already a few days after secondary immunization with the same antigen, however, the bone marrow appeared to be the major source of serum antibodies [6]. This bone marrow antibody formation coincided with the occurrence of B and T memory cells [7]. Peripheral lymphoid organs were directly involved in the initiation of bone marrow antibody formation by these memory cells [8].

Soon after my arrival at The Institute, Ivan invited me to review our data on antibody formation in the bone marrow for the Festschrift in Honour of Niels Jerne on the occasion of this 70th birthday [9] and to write a chapter on the methodological aspects of these studies for the second volume of his *Immunological Methods* [10]. Ivan's interest in the subject stimulated me to further dissect the phenomenon of antibody formation in the bone marrow. These studies, employing priming and boosting the immune system of mice with various combinations of hapten-carrier conjugates, showed that B memory cells, but not necessarily T memory cells were required for antibody formation in the bone marrow [11]. The origin of the antibody-forming cells, which appear in the bone marrow during secondary immune responses, were studied by Michael Julius, Dennis Osmond and myself in The Institute. In these studies, we made use of parabiotic mice consisting of previously immunized CWB and CSW mice or previously immunized BAB/14 and BALB/c mice. The members of both pairs (CWB versus CSW and BAB/14 versus BALB/c) had a different allotype of the Igh-1 locus. By using such allotype-different

parabiotic pairs and the analysis of the allotype of the antibodies produced in the bone marrow, we showed that bone marrow antibody formation is due to immigration of reactivated memory B cells from the spleen and other peripheral lymphoid organs, and that part of the bone marrow plasma cells have a long life-span [11].

In the eighties and nineties, more immunologists became interested in bone marrow antibody formation, and the subject became a regular part of the textbooks. In recent years, the groups of a.o. Noelle, Cyster, Radbruch, Zheng and Ahmed have considerably extended our insight into bone marrow antibody formation by identifying the precursors of short-lived and long-lived bone marrow plasma cells, and by delineating the role of cytokines, chemokine receptors and adhesion molecules in the recruitment and survival of these precursors and their progeny in the murine marrow [12–17]. In addition, the group of Yoshie presented evidence that similar chemokine receptors and adhesion molecules underly the recruitment of plasma cells to the human bone marrow [18].

Dissection of cell populations: frequencies of immunocompetent B and T cells

The identification of the lymphocyte as the basic functional unit of the immune system by Gowans, the clonal selection theory of Burnet, Jerne's plaque assay for identifying single antibody-producing cells, and Mishell and Dutton's method for induction of primary immune responses *in vitro* were the basis for Ivan's successful attempts to develop a limiting dilution culture system for frequency analyses of responsive lymphocytes [19]. By the end of the seventies, several groups in The Institute had adopted and adapted Ivan's technology in order to acquire really quantitative data on the structure and function of the immune system. By collaboration with Antonio Coutinho and the late Max Schreier, I got heavily involved in these clonal culture systems and frequency analyses. Together, we studied the immune competence of surface immunoglobulin (Ig)-bearing B cells in the bone marrow, employing three limiting dilution culture systems: (i) a specific helper assay with sheep red blood cells as antigen and using *in vivo* activated Th cells; (ii) a non-specific helper assay using Con A-induced factors as a source of help and (iii) polyclonal activation with lipopolysaccharide (LPS). It appeared that the frequencies of ν -gene expression in bone marrow B cells were of the same magnitude as the corresponding frequencies of splenic B cells. Bone marrow B cells appeared also fully susceptible to stimulation by antigen in combination with either specific or non-specific T-cell help as well as to stimulation by LPS [20]. Also the frequency of heavy-chain isotype switching from IgM to IgG1, IgG2a, IgG2b, IgG3 and IgA after LPS stimulation was the same in bone marrow B

cells and splenic B cells [21]. Interestingly, similar frequencies of *v*- and *c*-gene expression were found in bone marrow and splenic B cells of germfree and aged mice [22, 23]. This led us to conclude that there is no difference in immune competence between surface Ig-bearing B cells from bone marrow and spleen and that environmental antigenic pressure and aging do not affect the *v*-gene repertoire and the *c*-gene switching capacity at the level of the LPS-responsive B cell.

Meanwhile, and after returning to the Erasmus University of Rotterdam, I studied the suppressive effects of intravenous immunization with irradiated or non-irradiated allogeneic blood cells on T-cell function in mice. Such *i.v.* immunization could effectively suppress delayed type hypersensitivity and graft versus host reactivity against H-2 and non-H-2 alloantigens in mice. The *i.v.* immunization with alloantigens appeared to induce suppressor T cells [24]. This procedure was considered relevant to the so-called 'blood transfusion effect' in clinical kidney transplantation, referring to the much better prognosis of a transplanted allogeneic kidney in patients that were subjected to blood transfusion before receiving a kidney transplant.

Because the *in vivo* studies were so interesting, more insight was needed in the effect of the *i.v.* alloimmunization at the clonal level. To this end, one of Ivan's limiting dilution culture systems was most appropriate: together with Lucien Aarden, Ivan had developed a microculture system for evaluating frequencies of alloantigen-activated helper (Th) and suppressor T (Ts) cells [25].

The results employing this system for frequency analysis of Th and Ts cells after *i.v.* alloimmunization came to us as a surprise. It appeared that, contrary to our expectations, not only the Ts cell frequencies had increased, but the Th-cell frequencies also, and to a greater extent [26]. This emphasized the value of a truly quantitative analysis for measuring the effects of manipulation of the immune system, and confirmed again the rightness of Ivan's quantitative approach.

Dissection of molecules: human chorionic gonadotropin (hCG) as a source of immunoregulatory oligopeptides

During pregnancy, the maternal immune system is under tight control to prevent rejection of the foetal 'allograft'. Often, the mother is also found to have increased protection against the clinical symptoms of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Th cells are central in foetal allograft rejection, the clinical symptoms of rheumatoid arthritis and multiple sclerosis. Skewing of the T-cell system towards Th2 is considered beneficial for a favourable outcome of the pregnancy as well as keeping the clinical symptoms of these autoimmune diseases at bay [27]. Specifically, downregulation

of nuclear factor- κ B in Th1 cells is thought to be essential for human pregnancy success [28].

Characteristic for human pregnancy is the production of hCG, a glycoprotein hormone first purified from the urine of pregnant women in 1927 [29]. Since its detection, hCG forms the basis of all pregnancy tests, and in clinics, it is used to treat women with anovulation. However, non-pregnant women and males also produce hCG. Here, the pituitary gland accounts for low levels of hCG [30, 31]. Most of the hCG produced during pregnancy is produced by syncytiotrophoblasts in the placenta [32], the organ where cells and tissues of mother and child intensely meet and where immunomodulation is most needed to fight off rejection.

Human CG exhibits a variety of forms in serum and urine, including heterodimeric hCG consisting of an intact α and β subunit, heterodimeric hCG with peptide bond cleavages in its β -subunit known as nicked hCG, hCG β -core fragments composed of residues 6–40 disulfide bridged to residues 55–92, and intact α and β subunits derived from dissociation of hCG [33–37]. While the α subunit is identical to that in other members of the glycoprotein hormone family (e.g. TSH, FSH, LH), the β subunit is unique to hCG [34]. Only intact hCG stimulates the hCG receptor. During pregnancy, besides intact hCG, nicked hCG and hCG β -core fragments are the two major forms in serum and urine [33, 34], with an increasing proportion of nicked hCG to total hCG and hCG β -core in serum and urine during gestation [33]. Both nicked hCG and hCG β -core consist of a β chain with a defective loop 2: β -core fragment completely lacks this loop (residues 41–54), while nicked hCG has peptide bond cleavages between residues 44 and 52.

Several investigators have studied the effect of heterodimeric hCG and its variants on the immune system because of their putative role in preventing the rejection of the foetal allograft during pregnancy. However, nobody studied the possible immunological activity of the *in vivo* liberated peptide fragments originating from nicking of the sequence MTRVLQGVLPALPQ (residues 41–54) of loop 2 of the β -subunit of hCG, probably because peptides as small as a few amino acids generally are not supposed to have significant biological activity.

Mainly based on the known preferential cleavage sites [33–37], we (RB, NAK) synthesized the peptides MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ, as well as QVVC and VVC from the flanking COOH-side, and tested these peptides in a variety of biological systems. Each of these peptides displayed a remarkable set of biological activities ranging from modulation of angiogenesis, the inhibition of septic shock syndrome and the prevention of diabetes to the reduction of ischaemia-reperfusion damage [38–40]. This data led us to suggest that breakdown products of hCG as small as three or four amino

acid residues play a central role in pregnancy success [38]. The occurrence of hCG in non-pregnant individuals [30, 31] suggests to us that also the above hCG-related oligopeptides might constitute an active component in the regulation of inflammatory circuits and immunity. Apparently, proteases are not just non-specific degradative molecules, but, at least in the case of hCG, they can liberate small oligopeptides with a most relevant physiological activity. In this context, it is interesting that several parasites (such as *Trypanosoma brucei brucei*) and bacteria (such as *Bacillus anthracis*) bear similar sequences in particular proteins [39]. It might be that, after penetration of the epithelium, these microorganisms release such sequences into their surroundings to suppress the local inflammatory reaction of the host, in order to make their life comfortable.

In view of the expertise in proteomics that Ivan had built up in the nineties [41, 42], and of course because of his analytical mind and broad interest in biological systems, we went to Ivan to discuss these data. When, some time later, the closing of The Institute was announced, we invited Ivan and Hana to spend some months in Rotterdam, for more detailed discussions on the hCG-oligopeptide mystery. Luckily, Ivan came gladly, and Hana, equally looking forward to a Rotterdam experience, accompanied him. During his mini-sabbatical in the spring of 2002, the hCG-data were profoundly analysed and avenues for further studies were delineated.

The identified hCG-related regulating small oligopeptides may cross cellular membranes without requiring membrane-bound receptors [43]. Gene expression analyses and other studies taught us that these oligopeptides have a distinct regulating effect on the expression of genes involved in inflammatory pathways and immunity [38, 39]. In view of the central role of inflammatory pathways in a variety of physiological and pathophysiological processes [44–46], this may explain the broad therapeutic activity of the identified hCG-related regulating oligopeptides in the models tested [38–40].

As stated before [39], we do not believe that the case of regulation of gene expression by hCG-related small oligopeptides is unique in biology. It is becoming increasingly clear that the presently known approximately 500 proteases and protease-like molecules in mammals [47] enable highly specific hydrolysis of peptide bonds, dependent on their localization and the local conditions. We predict that further research on the dissection of proteins will unlock a wealth of small oligopeptides that can regulate many more physiological processes. Needless to say, should this be true, we foresee an equally large array of potential pharmaceutical molecules.

Dear Ivan, it was a privilege and pleasure to interact with you during all these years. Thank you from the bottom of my heart. *Rob*

References

- Oden ThC, ed. *Parables of Kierkegaard*. Princeton, New Jersey: Princeton University Press, 1978.
- Lefkovits I, ed. *A Portrait of the Immune System*. Scientific Publications of N.K. Jerne. Singapore: World Scientific Publishing Co, Pte. Ltd., 1996.
- van der Blom N. Rotterdam and Erasmus. In: Sperna Weiland J, Frijhoff WThM, eds. *Erasmus of Rotterdam. The man and the scholar*. Proceedings of the Symposium Held at the Erasmus University, Rotterdam. Leiden: Brill Academic Publications, 1988:240–52.
- Pfeiffer R, Marx. Die Bildungsstätte der Cholerascchutzstoffe. *Z Hyg U Infektionskrankh* 1898;27:272–97.
- Deutsch L. Contribution à l'étude de l'origine des anticorps typhiques. *Ann Inst Past* 1899;9:689–727.
- Benner R, Meima F, van der Meulen GM, van Ewijk W. Antibody formation in bone marrow. III. Effects of route of priming and antigen dose. *Immunology* 1974;27:747–60.
- Benner R, Meima F, van der Meulen GM. Antibody formation in bone marrow. II. Evidence for a memory-dependent phenomenon. *Cell Immunol* 1974;13:95–106.
- Benner R, van Oudenaren A, de Ruiter H. Antibody formation in bone marrow. IX. Peripheral lymphoid organs are involved in the initiation of bone marrow antibody formation. *Cell Immunol* 1977;34:125–37.
- Benner R. The bone marrow: a major site of antibody production. In: Steinberg CM, Lefkovits I, eds. *Festschrift in Honor of Niels Kaj Jerne on the Occasion of His 70th Birthday*. Basel: S Karger, 1981:362–71.
- Benner R, van Oudenaren A, Koch G. Induction of antibody formation in mouse bone marrow. In: Lefkovits I, Pernis B, eds. *Immunological Methods II*. New York: Academic Press, 1981:247–61.
- Koch G, Osmond DG, Julius MH, Benner R. The mechanism of thymus-dependent antibody formation in bone marrow. *J Immunol* 1981;126:887–90.
- Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* 1995;69:1895–902.
- Hargreaves DC, Hyman PL, Lu TT *et al*. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med* 2001;194:45–56.
- Han S, Yang K, Ozen Z *et al*. Enhanced differentiation of splenic plasma cells but diminished long-lived high-affinity bone marrow plasma cells in aged mice. *J Immunol* 2003;170:1267–73.
- Cassese G, Arce S, Hauser AE *et al*. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol* 2003;171:1684–90.
- Kunkel EJ, Butcher EC. Plasma-cell homing. *Nat Rev Immunol* 2003;3:822–9.
- O'Connor BP, Raman VS, Erickson LD *et al*. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med* 2004;199:91–7.
- Nakayama T, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O. Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. *J Immunol* 2003;170:1136–40.
- Lefkovits I, Waldmann H. *Limiting Dilution Analysis of Cells in the Immune System*. Oxford: University Press, 1999.
- Benner R, Rijnbeek A-M, Schreier MH, Coutinho A. Frequency analysis of immunoglobulin ν -gene expression and functional reactivities in bone marrow B cells. *J Immunol* 1981;126:887–90.
- Benner R, Coutinho A, Rijnbeek A-M, van Oudenaren A, Hooijkaas H. Immunoglobulin isotype expression. II. Frequency analysis in mitogen-reactive B cells. *Eur J Immunol* 1981;11:799–804.

- 22 Hooijkaas H, van der Linde-Preesman AA, Bitter WM, Benner R, Pleasants JR, Westmann BS. Frequency analysis of functional immunoglobulin c- and v-gene expression by mitogen-reactive B cells in germfree mice fed chemically defined ultra-filtered 'antigen-free' diet. *J Immunol* 1985;134:2223-7.
- 23 Hooijkaas H, Preesman AA, van Oudenaren A, Benner R, Haaijman JJ. Frequency analysis of functional immunoglobulin c and v gene expression in murine B cells at various ages. *J Immunol* 1983;131:1629-34.
- 24 Benner R, Wolters EAJ, Bril H, Molendijk A, van Oudenaren A. Regulation of delayed type hypersensitivity to host histocompatibility antigens during graft-versus-host reactions. *Immunol Rev* 1985;88:25-57.
- 25 Aarden L, Corley RB, Soederberg A, Lefkovits I. Limiting dilution analysis of the suppressive effect mediated by alloantigen-primed cells. *Immunology* 1980;41:399-406.
- 26 Bianchi ATJ, Schilham MW, Benner R, Young P, Lefkovits I. *In vivo* priming of helper and suppressor T cells by alloantigens. Frequency analysis with the use of an *in vitro* limiting dilution assay. *J Immunol* 1987;139:2524-9.
- 27 Sacks G, Sargent I, Redman C. An innate view of human pregnancy. *Immunol Today* 1999;20:114-8.
- 28 McCracken SA, Gallery E, Morris JM. Pregnancy-specific down-regulation of NF-kappa B expression in T cells in humans is essential for the maintenance of the cytokine profile required for pregnancy success. *J Immunol* 2004;172:4583-91.
- 29 Goretzlehner G, Rudolf K. 50 years ago - 1927 - the 1st demonstration of chorionic gonadotropin in the urine of pregnant women by Aschheim and Zondek. *Zentralbl Gynakol* 1978;100:638-41.
- 30 Yoshimoto Y, Wolfsen AR, Hirose F, Odell WD. Human chorionic gonadotropin-like material: presence in normal human tissues. *Am J Obstet Gynecol* 1979;134:729-33.
- 31 Birken S, Maydelman Y, Gawinowicz MA, Pound A, Liu Y, Hartree AS. Isolation and characterization of human pituitary chorionic gonadotropin. *Endocrinology* 1996;137:1402-11.
- 32 Amoroso EC, Perry JS. The existence during gestation of an immunological buffer zone at the interface between maternal and foetal tissues. *Philos Trans R Soc Lond B Biol Sci* 1975;271:343-61.
- 33 Cole LA, Kardana A, Park S-Y, Braunstein GD. The deactivation of hCG by nicking and dissociation. *J Clin Endocr Metab* 1993;76:704-10.
- 34 Alftan H, Stenman UH. Pathophysiological importance of various molecular forms of human choriongonadotropin. *Mol Cell Endocrinol* 1996;125:107-20.
- 35 Kardana A, Elliott MM, Gawinowicz MA, Birken S, Cole LA. The heterogeneity of human chorionic gonadotropin (hCG). I. Characterization of peptide heterogeneity in 13 individual preparations of hCG. *Endocrinology* 1991;129:1541-50.
- 36 Cole LA, Kardana A, Andrade-Gordon P *et al*. The heterogeneity of human chorionic gonadotropin (hCG). III. The occurrence and biological and immunological activities of nicked hCG. *Endocrinology* 1991;129:1559-67.
- 37 Birken S, Maydelman Y, Gawinowicz MA. Preparation and analysis of the common urinary forms of human chorionic gonadotropin. *Methods* 2000;21:3-14.
- 38 Khan NA, Benner R. Immunoregulator. European Patent Application EP00.201.139.3 (29 March 2000). International Patent Application PCT/NL01/00259 (29 March 2001, granted).
- 39 Khan NA, Benner R. Gene regulation by peptides. European Patent Application EP01.203.748.7 (4 October 2001). International Patent Application PCT/NL02/00639 (4 October 2002, granted).
- 40 Khan NA, Khan A, Savelkoul HFJ, Benner R. Inhibition of septic shock in mice by an oligopeptide from the β -chain of human chorionic gonadotrophin hormone. *Hum Immunol* 2002;63:1-7.
- 41 Lefkovits I, Kettman JR, Coleclough C. A strategy for founding a global lymphocyte proteinpaedia and gene catalogue. *Immunol Today* 1990;11:157-62.
- 42 Frey JR, Kuhn L, Kettman JR, Lefkovits I. The amino acid composition of 350 lymphocyte proteins. *Mol Immunol* 1994;31:1219-31.
- 43 Futaki S, Goto S, Suzuki T, Nakase I, Sugiura Y. Structural variety of membrane permeable peptides. *Curr Protein Pept Sci* 2003;4:87-96.
- 44 Nathan C. Points of control in Inflammation. *Nature* 2002;420:846-91.
- 45 Oyama J, Blais C, Liu X *et al*. Reduced myocardial ischemia-reperfusion injury in Toll-like receptor 4-deficient mice. *Circulation* 2004;109:784-9.
- 46 Ishii M, Suzuki Y, Takeshita K *et al*. Inhibition of c-Jun NH₂-terminal kinase activity improves ischemia/reperfusion injury in rat lungs. *J Immunol* 2004;172:2569-77.
- 47 López-Otin C, Overall CM. Protease degradomics: a new challenge for proteomics. *Nature Rev Mol Cell Biol* 2002;3:509-19.