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Induction of Autoimmunity Through Bystander Effects. Lessons from Immunological Disorders Induced by Heavy Metals

Gilbert J. Fournié, Magali Mas, Bastien Cautain, Magali Savignac, Jean-François Subra, Lucette Pelletier, Abdelhadi Saoudi, Dominique Lagrange, Maryline Calise and Philippe Druet

Institut National de la Santé et de la Recherche Médicale (INSERM) U28, Institut Fédératif de Recherche (IFR) 30, Hôpital Purpan and Université Paul Sabatier, Toulouse, France

Autoreactive T cells exist in healthy individuals and represent a potential reservoir of pathogenic effectors which, when stimulated by microbial adjuvants, could trigger an autoimmune disease. Experimental studies have indicated that xenobiotics, well defined from a chemical point of view, could promote the differentiation of autoreactive T cells towards a pathogenic pathway. It is therefore theoretically possible that compounds present in vaccines such as thiomersal or aluminium hydroxyde can trigger autoimmune reactions through bystander effects.

Mercury and gold in rodents can induce immunological disorders with autoimmune reactions. *In vitro*, both activate signal transduction pathways that result in the expression of cytokines, particularly of IL-4 and IFNγ. In a suitable microenvironment heavy metals could therefore favour the activation of autoreactive T cells. In that respect, genetic background is of major importance. Genome-wide searches in the rat have shown that overlapping chromosomal regions control the immunological disorders induced by gold salt treatment, the development of experimental autoimmune encephalomyelitis and the $CD45RC^{high}/CD45RC^{low}$ $CD4^+$ T cells balance. The identification and functional characterization of genes controlling these phenotypes may shed light on key regulatory mechanisms of immune responses. This should help to improve efficacy and safety of vaccines.

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Key words: bystander effect, adjuvants, autoimmunity, gold salts, mercury

Introduction

The normal immune response to pathogens usually requires adjuvant effects mediated by microbial compounds that lead to macrophage activation through various mechanisms. This activation plays a major role in the first line of defense against common microorganisms and also play a crucial role in the initiation and development of adaptative immune responses.

Adjuvants are also critical components of vaccines which are commonly used in human and veterinary medicine to induce prophylactic immunization. However, their precise modes of action are often poorly understood. They might theoretically play a role at one or several steps of the cascade of events involved in the development of an immune response either triggered by an infectious agent or by a vaccine [1] (Figure 1). First of all, they may facilitate antigen uptake, transport and presentation by antigen presenting cells (APCs). At that stage, adjuvant could be important to increase attraction of dendritic cells towards the injection site, to increase loading of APCs and to increase transport of antigen-loaded APCs towards the lymph node. Adjuvant could also favour the release of antigen to lymphoid tissues through the so called 'depot effect'. Another mechanism could be the activation of innate immune cells triggered by pathogen-recognition receptors, responsible for the secretion of cytokines and the secondary activation of APCs. Acting as or inducing a 'danger signal' from damaged tissues, some adjuvants could also trigger the upregulation of co-stimulatory molecules on APCs. All these potential mechanisms emphasize the key roles of adjuvants on secretion of cytokines and thereafter on APCs activation and on upregulation of co-stimulatory molecules.

The relationship between infectious illnesses of bacterial, viral or parasitic origin and the subsequent development of autoimmune diseases has been put

Correspondence to: Gilbert J. Fournié, INSERM U28, Hôpital Purpan, Pavillon Lefebvre, place du Dr Baylac, 31059 Toulouse cedex, France. Tel.: 33-5-61-77-92-93. Fax: 33-5-61-77-92-91. E-mail: gfournie@purpan.inserm.fr

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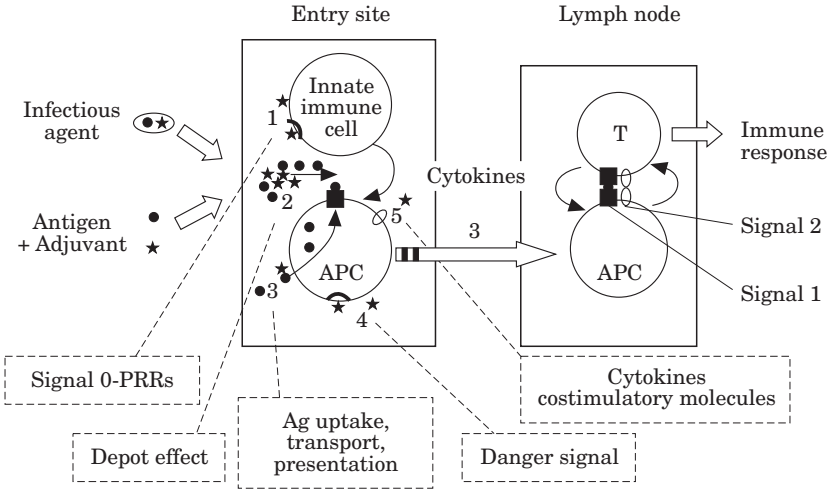

Figure 1. Different possible modes of action of adjuvants. 1. Activation of innate immune cells through signaling of pathogen-recognition receptors (PRRs) constitutively expressed on cells from the innate immune system. 2. Depot effect favoring prolonged antigen release and presentation. 3. Antigen uptake and presentation by antigen presenting cells (APCs). Transport of antigen-loaded APCs towards the lymph node. 4. Induction of a danger signal following tissue destruction or stress. 5. Cytokines and co-stimulatory molecules (0) are the critical communication signals for the induction of the immune response through the simultaneous delivery of signal 1 and signal 2.

forward in many studies [2]. The question whether vaccines can cause immunological disorders including autoimmunity has been recently raised in numerous reports. The mechanisms by which infectious agents on the one hand and vaccines on the other hand could trigger the development of autoimmune diseases are poorly understood but could be similar in both instances. Molecular mimicry is very often the favoured mechanism [3, 4]. However an additional and non-exclusive possibility exists that adjuvant can trigger the development of autoimmunity through bystander effects. This paper is devoted to this question.

In the first part we discuss the basis for such a possibility, namely the presence in healthy individuals of autoreactive immune cells. We then present data demonstrating that xenobiotics could actually behave as adjuvants to promote the differentiation of autoreactive T cells towards pathogenic pathways. The following parts will show that mercury, an additive frequently associated to antigens in vaccines, as well as gold another heavy metal, can trigger in experimental conditions the development of immunological disorders and of autoantibodies. Both metals can activate signal transduction pathways that lead to the secretion of cytokines indicating that their effects on the immune system can mimic to some extent that ones of adjuvants able to mediate bystander effects. Finally we will focus on the potential importance of genetic factors that could be at play in the development of autoimmunity through bystander effects. Indeed recent studies in the rat indicate that the same chromosomal regions control the development of experimental autoimmune encephalomyelitis and of the immunological disorders triggered by gold salts, and suggest that these controls could be mediated by some CD4+ T cell sub-populations.

Autoreactive T Cells Do Exist in Healthy Individuals and Represent a Potential Reservoir of Pathogenic Effectors

It is now well established that autoreactive T and B cells circulate in the blood of healthy individuals [5]. Under normal circumstances an immunoregulatory network is likely to protect the host against autoreactive cells. As far as the T cell compartment is concerned, regulatory T cells have been described in various experimental models of autoimmune diseases. In the rat particularly, a subset of $CD4^+$ T cells expressing a low level of expression of the CD45RC marker has been found to exhibit a regulatory function on pathogenic autoreactive T cells [6, 7]. Congenital athymic rats injected intravenously with $CD45RC^{high}$ $CD4^+$ T cells, from histocompatible congenic euthymic donors, develop a severe wasting disease characterized by multi-organ mononuclear cellular infiltration. The cotransfer of $CD45RC^{low}$ with the $CD45RC^{high}$ subset prevents the appearance of the wasting disease. These data show that the normal T cell repertoire contains pathogenic T cells ($CD4^+CD45RC^{high}$) and regulatory T cells ($CD4^+CD45RC^{high}$) and that this latter population has a dominant effect in regulating the former. Similar results were obtained in SCID mice [8]. These data demonstrate that potentially autoaggressive Th1 cells are present in normal conditions and are controlled by regulatory T cell subpopulations.

Under the pathogenic conditions of infectious diseases, autoreactive T cells could be activated and could trigger the initiation and development of autoimmune diseases. Both specific antigens and compounds with adjuvant properties present in the infectious agents could play some role. It is important to

keep in mind that in pathological situations, the sequence of events leading to autoimmunity could be dependent on several mechanisms. The molecular mimicry hypothesis suggests that similar epitopes shared by the pathogen and the host can activate autoreactive T cells. In a first step the response to a dominant protein of the infectious particle may activate T cell that cross-react with cryptic self epitope. Thereafter the mechanism of determinant spreading might be at work in the development of the autoimmune disease [4]. Specific antigens of the pathogen can also play the role of superantigen and be responsible either for the activation of autoreactive T cells or for the deletion of regulatory T cells [9, 10]. The adjuvant properties of infectious agents can also, as indicated in the introduction, play a role in the various steps of an immune response, and could therefore participate in the activation of autoreactive T cells. Analysing the effect of xenobiotic well defined from the biochemical point of view reveals that such a possibility actually exists.

Xenobiotics Acting as Adjuvants to Elicit Protective Th1 Immunity Could also Promote the Differentiation of Autoreactive T Cells Towards a Pathogenic Pathway

Several attempts have been made to define the components of whole microbial preparations that are responsible for adjuvant effects in order to further develop new adjuvants that could be used in vaccines. Some of the adjuvant properties of the bacterial walls of gram-negative bacteria have been clearly attributed to the Lipid A fraction of lipopolysaccharides [11]. Similarly muramyl-dipeptide has been shown to be the smallest peptidic moiety of bacteria cell walls that can replace mycobacteria in Freund's complete adjuvant [12, 13].

More recently, interest has been focused on another well defined structure endowed with adjuvant activity found in bacterial DNA. Studies on immunomodulatory properties of bacterial DNA have shown that unmethylated CpG motifs displaying 5′ Pu-Pu-CpG-Pyr-Pyr 3′ (Pu: purine, A or G; Pyr: pyrimidine, C or T) nucleotide sequences are recognized by, and can activate, cells of the immune system [14]. Such motifs allow to discriminate pathogen-derived foreign DNA from self DNA. CpG motifs were found to activate antigen-presenting cells leading to up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules, and to the secretion of pro-inflammatory cytokines particularly TNF-α, IFN-γ, IL-1, IL-6, IL-12 and IL-18 [15, 16]. CpG oligonucleotides thus act as adjuvants that switch on T-helper 1 (Th1) immunity and are potential adjuvants for human vaccines to elicit protective Th1 immunity [17] even in human infants [18]. They could also be used in Th-2 driven diseases, to redirect the immune system towards a curative Th1 response [19]. However the mode of action of such adjuvants suggests that in predisposed individuals, they could trigger Th1-mediated autoimmune diseases through a bystander effect. This possibility has been recently investigated in an experimental mouse model of experimental allergic encephalomyelitis (EAE) [20].

EAE represents a widely used model of multiple sclerosis in humans. It is induced by injection of a target autoantigen (a peptide of the myelin basic protein) emulsified in the complete Freund's adjuvant that contains heat-killed mycobacteria. Segal and co-workers first demonstrate that CpG-containing oligodeoxynucleotides (ODN) can substitute for mycobacteria in the priming of encephalitogenic myelin-reactive T cells and for the induction of disease *in vivo*. When mice are immunized with the same preparation but lacking mycobacteria or CpG ODN, disease does not develop. In that particular situation, T cells appeared to be in a transitional state characterized by expression of low levels of the IL-12Rβ2 subunit. These T cells behave as 'pre-Th1' cells and retained the ability to differentiate into encephalitogenic effectors when reactivated under Th1-polarizing conditions, through an IL-12-dependent upregulation of the IL-12Rβ2 subunit [20].

While these results confirm the adjuvant power of CpG ODN, they also suggest that they could potentially trigger autoimmune diseases in a susceptible individual. It is possible that in healthy individuals, populations of autoreactive T resembling pre-Th1 cells do exist. Indeed, as other autoantigens, some myelin antigens are expressed in peripheral sites [21] and pre-Th1 cells could be sensitized following an activation in a noninflammatory setting and could persist thereafter as memory cells. Such T cells could fully differentiate into pathogenic Th1 effectors under reactivation in an inflammatory milieu during infectious illnesses or vaccination if activated APCs loaded with the sensitizing autoantigen are present [22] (Figure 2). Every compound added to a vaccine preparation should therefore be considered for its potential ability to favour the switching of presensitized T cells to pathogenic T cells.

Mercury and Gold Salts Induce in Susceptible Rats Immunological Disorders Characterized by Autoimmune Reactions

Mercury is a heavy metal that is an additive commonly found in vaccine preparations as thiomersal. Administration of subtoxic doses of mercuric chloride induces in susceptible Brown Norway (BN) rats immunological disorders characterized by a sharp IgE response, the production of autoantibodies, particularly of anti-DNA and anti-laminin antibodies, and the development of a glomerulopathy. Rats display linear IgG deposits along the glomerular basement membrane and develop proteinuria [23–25]. They exhibit a

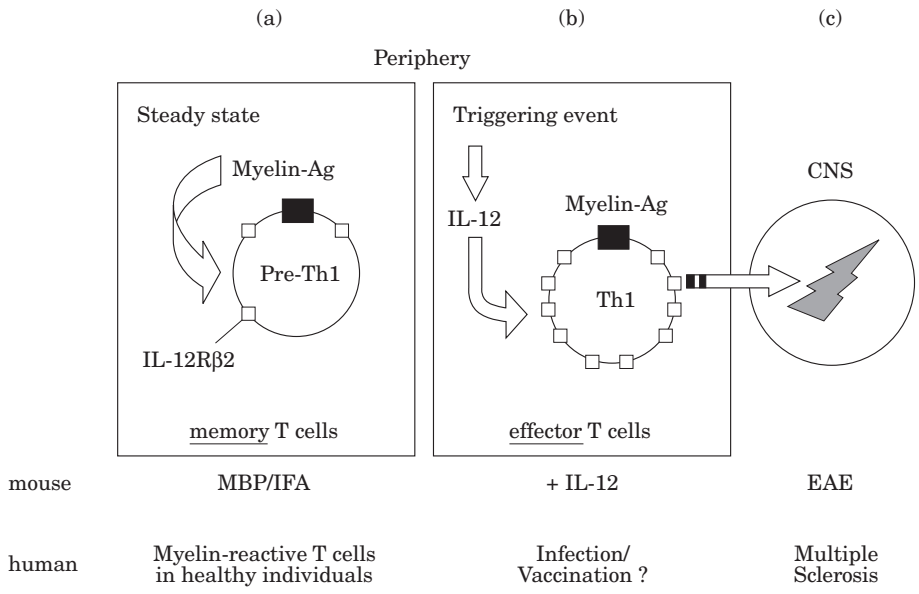

Figure 2. Hypothetical mechanism for the triggering of autoimmune disease in susceptible individuals. This mechanism is shown for experimental autoimmune encephalomyelitis (EAE) in the mouse, a model of multiple sclerosis. Experimental results that support such a mechanism (line: 'mouse') are from [20]. (a) In normal conditions, myelin basic protein (MBP)-reactive T cells exist in the periphery in a 'pre-Th1' state characterized by expression of the IL-12Rβ2 subunit. (b) Under a triggering event such as an infection or a vaccination, the secretion of IL-12 triggers the up-regulation of IL-12Rβ2 subunit and the differentiation of these T cells into pathogenic effector cells able to migrate (c) into the central nervous system (CNS).

$CD4^+$ T cell-dependent polyclonal activation of B cells. In contrast, Lewis (LEW) rats are resistant and develop an immunosuppression mediated by $CD8^+$ T cells recruited by $CD4^+$ T cells [26]. Gold salts induce the same immunological disorders in BN rats. Lewis rats are resistant but do not develop immunosuppression under gold salt administration [27–29].

Autoreactive T cells found in BN rats injected with mercury or gold salts have a Th2 phenotype particularly characterized by IL-4 secretion. T cell lines derived from gold salt injected BN rats promote B cell polyclonal activation *in vitro* and transfer the disease into $CD8^+$ cell-depleted BN recipients. This suggests that $CD8^+$ cells are normally able to counteract pathogenic autoreactive T cells and that disease induced by gold salts or $HgCl_2$ is due to both the emergence of autoreactive Th2 cells and to a defect at the $CD8^+$ level. By contrast, T cells that expand in the LEW resistant strain produce IFN-γ and TGF-β and have protective effects on both Th1- [26] and Th2-autoimmune diseases [30].

In vitro studies have shown that, under exposure to $HgCl_2$, purified T cells from BN and LEW rats expressed increased production of IFNγ mRNA while only purified T cells from BN rats express IL-4 mRNA [31]. Therefore, according to the genetic background of the strain, a given xenobiotic could trigger different levels of expression of cytokines with quite different consequences on *in vivo* immune responses. These observations prompted us to investigate the mechanism(s) of action of mercury and gold salts on cytokine gene expression, and the genetic control of *in vivo* responsiveness to gold salts.

Mercury and Gold Salts Activate Signal Transduction Pathways that Trigger the Expression of IL-4 and IFNγ

Using murine T cell hybridomas that express IL-4 mRNA upon stimulation with $HgCl_2$, it was found that $HgCl_2$ induces a protein kinase C-dependent Ca^{2+} influx through L-type calcium channels. Calcium/calcineurin-dependent pathway and protein kinase C activation were both implicated in $HgCl_2$-induced IL-4 gene expression [32]. Moreover, *in vitro* it was found that $HgCl_2$ can activate directly protein kinase C.

Investigations were more recently conducted on the molecular targets of gold salts. It was found that, in T cells from BN and LEW rats, gold salts act on early steps of signal transduction, triggering a calcium signal. They induce tyrosine phorphorylation of numerous proteins including $p56^{lck}$. However, both *in vitro* and *in vivo*, the IL-4 response was favoured in cells from BN rats as compared to those from LEW rats (Savignac *et al.*, submitted). In this study it was also found that IFNγ production contributes at least in part to the resistance of LEW rats to gold salts-induced immunological disorders. Taken together, these results indicate that, mercury and gold salts behave as adjuvants, acting on T cell independently of antigen-specific recognition and triggering expression of cytokines. The adjuvant effect of mercury salts on the production of ovalbumin-specific IgE antibodies had been previously described [33]. Differential effects observed in cells from BN and LEW origin are

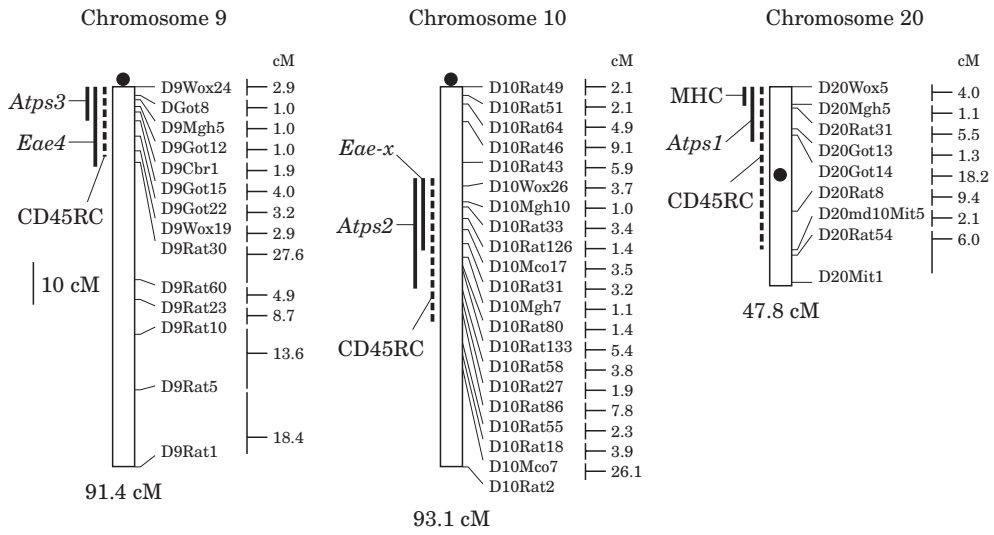

Figure 3. Diagrammatic representation of chromosomes and location of susceptibility loci on chromosomes 9, 10 and 20. Genetic maps were constructed using data collected on (LEW×BN) F2 hybrids rats. On the right of each chromosome are indicated the markers and the distance in centiMorgans (cM). The positions of the centromeres are shown as closed circles. Vertical bars on the left of each chromosome show the position of various susceptibility loci that have been described in these regions. *Atps1, Atps2* and *Atps3* are loci for susceptibility to develop IgE response in rats injected with aurothiopropanol sulfonate (*Atps*) [34]. *Eae4* and *eae-x* are loci for susceptibility to experimental autoimmune encephalomyelitis (EAE). The locus for EAE susceptibility described on chromosome 10 has not yet been named and is therefore indicated *Eae-x*. Shaded bars indicated CD45RC represent the chromosomal regions that have been found to control the percentage of the $CD45RC^{high}$ CD4 T cell population.

dependent on genetically controlled differences in these two strains. Under such an adjuvant activation, the genetic background could favour a Th2 type response in BN rats and a Th1-type response in LEW rats. These differences would reflect the differences between BN and LEW rats with respect to the polarization of their immune responses and to their susceptibility to develop immunological disorders.

The Same Genomic Regions Control the Th2-mediated Gold Salt Response, the Th1-mediated Susceptibility to Experimental Autoimmune Encephalomyelitis and the $CD45RC^{high}/CD45RC^{low}$ CD4+ T cell Subpopulations in the Rat

Genome-wide search for loci controlling the immunological disorders triggered by gold salts in the rat have identified three susceptibiltiy loci on chromosomes 9, 10 and 20, respectively named *Atps3* (Atps: aurothiopropanol sulfonate), *Atps2* and *Atps1* [34]. *Atps1* contains the MHC region that is associated with the susceptibility to the development of autoimmune diseases in rat, mouse and human, particularly to experimental autoimmune encephalomyelitis in rodents and to multiple sclerosis in humans. It was also noted with interest that independent genome-wide searches for loci controlling the susceptibility to EAE have identified in the rat overlapping regions to *Atps2* and *Atps3* on chromosome 10 (LEW×BN crosses) [35] and 9 (DA×BN crosses) [36] (Figure 3). On chromosome 10, these regions of susceptibility contain a cytokine gene cluster that bears several candidate genes including IL-4 IL-5, IL-3, IFN-regulatory factor-1 genes [37]. In man, this cluster on the long arm of human chromosome 5 in 5q31.1 has been linked to serum IgE concentrations in families of atopic patients from different ethnic origins [38, 39]. In the mouse, *Tpm1*, a locus that controls *in vitro* the Th1/Th2 differentiation has also been localized in the homologous region of the cytokine gene cluster on chromosome 11 [40].

Allelic variants of genes at these loci found on chromosomes 9, 10 and 20 could therefore be implicated in the control of T cell polarization to either a Th1 or a Th2 type of immune response. Such a possibility would fit with the observation that BN rats are prone to develop Th2-mediated diseases and resistant to the development of experimental autoimmune encephalomyelitis, while the LEW and the DA rats are prone to develop Th1-mediated autoimmune diseases and resistant to gold salt induced immunological disorders. In this respect it should be mentioned that the prevalence of Th2-mediated allergic diseases is decreased in patients suffering from multiple sclerosis [41].

This hypothesis has been strengthened in recent investigations on the factors that influence the balance between the T-helper subsets in rats as defined by the expression of CD45RC. It was already known that LEW and DA rats have a preponderance of $CD45RC^{high}$ T cells while in BN rats the $CD45RC^{low}$ CD4 T cells predominate ([42] and unpublished observations). However, there was no direct evidence that the $CD45RC^{high}/CD45RC^{low}$ ratio within the CD4 T cell compartment was involved in the susceptibility

to develop autoimmunity. It was recently shown [43] that, upon stimulation in an APC independent system, the anti-inflammatory cytokines IL-4, IL-10 and IL-13 are almost exclusively produced by the $CD45RC^{low}$ sub-population of CD4 T cells and that the difference in $CD45RC^{high}/CD45RC^{low}$ ratio between LEW and BN rats is intrinsic to haematopoietic cells. A genome-wide search for quantitative trait loci controlling the $CD45RC^{high}/CD45RC^{low}$ ratio was then performed in an F2 intercross between LEW and BN rats. The striking result obtained was that the regions associated with the control the $CD45RC^{high}/CD45RC^{low}$ CD4 T cell balance phenotype were located on chromosomes 9, 10, and 20 in regions that overlap with regions associated with susceptibility to gold salt-induced immunological disorders in BN rats and to EAE in LEW or DA rats. Moreover, as expected, a significant correlation was found in the F2 population between the $CD45RC^{high}/CD45RC^{low}$ ratio and the Th2-response induced by gold salts injections. Since the $CD45RC^{low}$ CD4 T cell subset has been shown to control inflammatory and autoimmune diseases, the balance between these $CD45RC^{high}$ and $CD45RC^{low}$ sub-populations may contribute to the susceptibility and severity of Th1- and Th2-mediated diseases. The identification and functional characterization of genes in the chromosomal regions identified may shed light on key regulatory mechanisms of immune responses that could be of major interest, particularly for vaccine strategy.

Conclusion

Vaccination has proven to be remarkably efficient in preventing infectious diseases in both human and veterinary medicines. While, in the past, numerous reports have led to the conclusion that vaccination is safe in patients with autoimmune diseases, questions have been recently raised concerning the possibility that vaccines could trigger autoimmune diseases. To our knowledge no controlled trial has yet confirmed such a possibility. However, it should be considered that in some predisposed individuals, immunization can trigger the development of autoimmunity through a bystander effect as suggested by experimental results.

Advances in biochemistry and molecular biology will undoubtedly in the future improve vaccine strategy. Understanding the genetic control of the regulatory mechanisms involved in immune responses and in autoimmunity will simultaneously help to improve efficacy and safety of vaccines based, if necessary, on screening of individuals at risk to develop adverse effects.

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