The use of recombinant-DNA techniques and hybridomas to make antibody(-fragments) that specifically recognize well defined dioxins and dibenzofurans in a competitive ELISA.

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Introduction

In the Netherlands (and probably elsewhere) there is a need for the rapid analysis of the dioxin/dibenzofuran content of food- and environmental samples. For TCDD, the use of the monoclonal antibody DD3 has shown to be a cheap and rapid alternative to GC/MS detection[1]. Although GC/MS remains the most sensitive technique, antibodies can be used to preselect polluted samples from unpolluted ones saving time and money. To perform such preselections, one must have access to specific antibodies. The aim of our study is to generate such a set of antibodies, each specifically recognizing one of the most toxic dioxin congeners.

Hybridomas

Up to now the anti-dioxin antibodies have been prepared using the hybridoma technique[2] where spleen cells of immunized mice are fused with myeloma-cancer cells resulting in hybridoma cell lines that are selected on the basis of the antibody they secrete. For the well defined DD3 antibody a total number of nearly a thousand hybridoma's has bee screened. These large numbers of hybridoma's are near the limit of what can reasonably be screened using standard immunochemical assays.

Phage-antibodies

Recent developments in molecular biology enable us to generate antibodies in a different way[3]. With immunoglobulin gene specific oligonucleotides, genes coding for immunoglobulins can be amplified by PCR and cloned into phagemid vectors. These vectors can subsequently be used to either generate large quantities of antibody that are secreted by bacteria, or the antibody can be expressed on the surface of a phage carrying the antibody gene inside its own DNA. The advantages of these recombinant DNA approaches are multiple. On one hand, the immunoglobulin genes of existing anti-dioxin hybridomas can be

studied and modified. This yields information on the rationale for antibody-antigen binding and enables the preparation of antibodies that show an improved binding. This is done by site-directed mutagenesis and induces small genetic modifications of the antibody genes and hence of the antibody structure. This may very well lead to an antibody with an improved antigen binding capacity that is prefered in antigen detection assays.
Besides using hybridomas to donate their immunoglobulin gene, it is possible to use the total cell population present in the spleen of an immunized mouse. When mRNA is extracted from these cells and converted into cDNA, the immunoglobulin specific oligonucleotide primers anneal to immunoglobulin sequences. In the following PCR reaction only these sequences are selectively amplified. These sequences are derived from approximately 10^8 different B-lymphocytes and code for as many different antibodies or antibody fragments. Selection of the resulting immunoglobulin-library is done using the phage display technology that is recently developed[4]. Phage are prepared that display the antibody proteins on their surface as fusion proteins to the phage gene 3 protein. To select for specific high affinity antibodies, a procedure called 'panning' is executed. Antibody expressing phages (approximately 10^{13}) are incubated with an antigen-coated surface. After extensive washing, only the high affinity antibodies expressing phages (10^{5-7}) are eluted from the surface. These can be used to grow antibody-producing bacteria. The eluted phages are however also used to again infect bacteria and to yield a new large population (> 10^{13}) of antigenbinding phages by rescuing with wild type phage VCSM13. The amplified first round phages are used in a second round of panning. After 3-5 round of panning most of the eluted phages represent good binding antibodies[4].

Antigens

Our investigations mainly focus on the preparation of antibodies to specific dioxin-haptens. Sofar we have used two conjugates to immunize Balb/c mice. These are BSA-TCDD and RSA-PCDD (fig. 1).

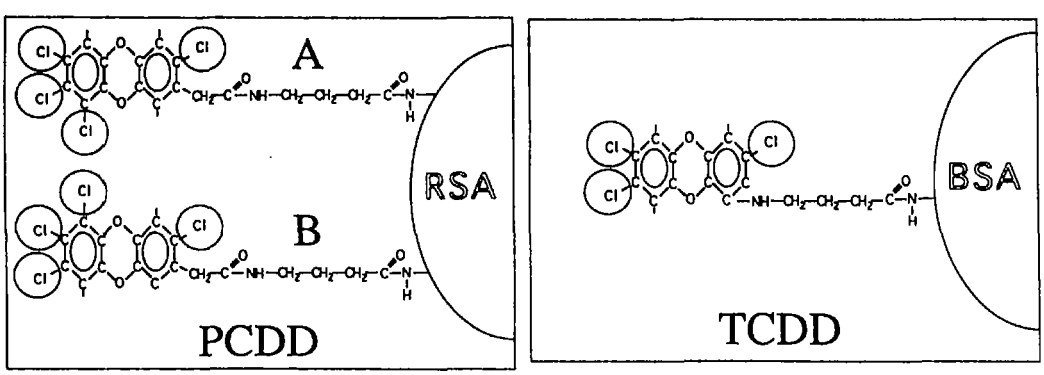

Fig. 1. Antigens used to immunize mice and to select hybridomas and phage antibodies. PCDD (left panel) equal mixture of two tetrachloro-dibenzo-p-dioxins (A and B) conjugated to rabbit serum albumin (RSA) using an adipine linker. TCDD (right panel).Conjugate of 1-amino-3,7,8-triCDD[1] and bovine serum albumin (BSA) using an adipine linker.

Results hybridomas

Using the hybridoma technique we prepared 2 hybridomas (A1 and A2) each recognizing the BSA-TCDD conjugate. However, A1 and A2 show no immunochemical reaction with BSA nor RSA but do appear to react the RSA-PCDD conjugate. We therefore hypothetize that these antibodies recognize an epitope that is not present on the carrier proteins but that is common in both dioxin-linker moieties of the immunogens.

We also isolated two hybridoma's (P1 and P2) that specifically recognize the RSA-PCDD conjugate and do not react with the BSA-TCDD conjugate. These hybridoma's have been characterized to a certain extent. They do not react with RSA nor with BSA. Both antibodies appear to react with an epitope that is specific for PCDD and that is not present on TCDD. Therefore we hypothetize that this epitope includes the chlorine atom adjacent to the oxygen atom in the PCDD molecules (cf. fig. 2) and possibly also the oxygen atom itself. To test this hypotheses we performed some preliminary competitive ELISA experiments with the RSA-PCDD conjugate as coating antigen and either the 2,3,4,7,8-PCDF (1) or the 1,2,3,7,8-PCDF (2) as competitor. Since these two dibenzofuran competitors are very similar to each other and differ only in the presence of the chlorine atom adjacent to a single oxygen atom, inhibition by competitor 1 sustains the hypothesis that both the chlorine adjacent to the oxygen atom as well as the oxygen atom itself are part of the recognized epitope. In fact for hybridoma P1 this appears to be true. One nanogram of competitor 1 resulted in a nearly complete inhibition of P1 binding the coating antigen.

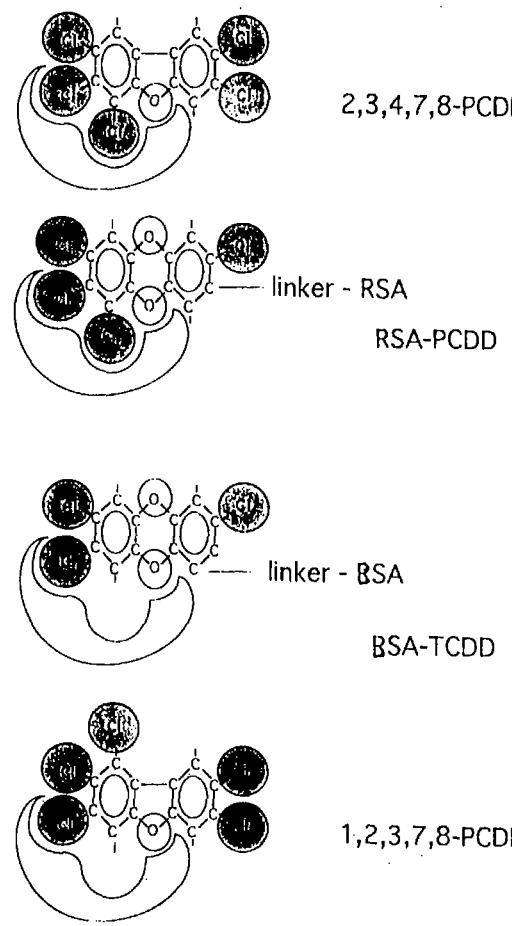

Fig. 2. Schematic representation of the epitope present on certain dioxins and dibenzofurans as it is suggested to be recognized by hybridoma P1.

Competitor 2 also showed inhibition in relation to P1 but at concentrations that are 20 times higher. The similarity between both competitors renders it acceptable that high doses of both compounds will react with the antibodies. Hybridoma P2 appears to be less sensitive to inhibition by the competitors. Further characterization of P1 and P2 is neccesary to elucidate the precise nature of the recognized epitopes.

Results Phage-antibodies

The phage display technique was used to prepare a phage library of immunoglobulins in the pCOMB3 phagemid[5]. The amplified immunoglobulin heavy and light chains were prepared from the spleen mRNA of a Balb/c mouse that had been injected with RSA-PCDD. The DNAs coding for the the immunoglobulin chains were cloned into pCOMB3 and a total number of 10^7 phagemids were electroporated into E. coli bacteria. These were used to prepare 10^{12} immunoglobulin expressing phages, each of which expresses a Fab-fragment of an IgG-immunoglobulin. After a first round of selection we eluted 10^7 phage from a RSA-PCDD-coated surface. These first round binders are used in subsequent panning rounds. The fact that bound phages could be eluted from the surface indicates the presence of RSA-PCDD-binders in the original library. We expect to select individual binders to the PCDD molecule after three or four rounds of panning. Moreover, we plan to use other dioxin- and furan-conjugates to extract binding phages from the initial library.
A still different approach we use at present is the use immunoglobulins of non-immunized (naive) human immunoglobulin library. Marks[6] has prepared such a library from human peripheral blood lymphocytes. He chose not to make phages carrying Fab fragments but prepared phages expressing only the antigen binding domains of immunoglobulins. These Fv-fragments of heavy and light chain were linked together to make a single chain Fv library (scFv). After two rounds of panning about 4 percent of the eluted phages are binding specifically to RSA-PCDD in a dose-dependent manner. No reaction with those binders could be observed with RSA or BSA alone and it is therefore most likely that the scFv phage-antibodies recognize the PCDD-linker complex. The specificity of the new phage antibodies is presently being investigated.

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