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Chlordecone potentiates auto-immune hepatitis and promotes brain entry of MHV3 during viral hepatitis in mouse models

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ABSTRACT

Chlordecone is an organochlorine used in the 1970's as a pesticide in banana plantations. It has a long half-life in the soil and can potentially contaminate humans and animals through food. Chlordecone targets, and mainly accumulates in, the liver, leading to hepatomegaly and neurological signs in mammals. Chlordecone does not cause liver injuries or any inflammation by itself at low doses, but it can potentiate the hepatotoxic effects of other chemicals and drugs. We studied the impact of chlordecone on the progression of acute hepatitis in mouse models of co-exposure to chlordecone with Concanavalin A or murine hepatitis virus type 3. We examined the progression of these two types of hepatitis by measuring hepatic transaminase levels in the serum and inflammatory cells in the liver, liver histological studies. Amplified tremors presented in the MHV3- chlordecone mouse model had led us to study the expression of specific genes in the brain. We show that chlordecone amplifies the auto-immune hepatitis induced by Concanavalin A by increasing the number of liver NKT cells, which are involved in liver damage. Chlordecone also accelerated the death of mice infected by murine hepatitis virus and enhanced the entry of the virus into the cervical spinal cord in infected mice, leading to considerable neurological damage. In conclusion, chlordecone potentiates both the Concanavalin A-induced hepatitis and brain damage caused by an hepatotropic/neurotropic virus.

1. Introduction

The liver is a vital organ subjected to various insults, including chemical agents, such as xenobiotics or drugs, viruses, and parasites. These agents can induce acute and/or chronic hepatitis. For example, Hepatitis A (HAV) and E (HEV) viruses cause acute, usually self-limiting, illnesses, whereas hepatitis B (HBV), C (HCV), and D (HDV) viruses induce both acute and chronic disease (Ponde, 2017). Repeated liver insults over a long period can result in hepatocyte death, inflammation, and fibrosis, leading to liver failure or the onset of liver cancer. Chlordecone is an organochlorine that was used primarily for agricultural purposes. It is a persistent environmental pollutant and its manufacture and use has led to significant contamination in the United States and the French West Indies that has adversely affected the environment and humans. Among the xenobiotics that affect the liver, chlordecone was shown to induce hepatomegaly in humans and animals in limited occupational and many experimental toxicological studies (Egle et al., 1978; Guzelian, 1982). This pesticide can also induce neurological damage in humans, manifested by convulsions, irritability, and tremors (Cannon et al., 1978; Taylor et al., 1978). The same effects have also been shown in rodent toxicological studies (Huang et al., 1981).

The liver is the main target of chlordecone, where it accumulates due to its ability to bind to plasma lipoproteins, which are responsible for the transport of cholesterol to the liver (Skalsky et al., 1979; Soine et al., 1982). The role of the liver in detoxifying molecules makes it a primary target of toxicity. Chlordecone combined with certain molecules can potentiate their hepatotoxicity, as shown for chloroform (Iijima et al., 1983), acetaminophen (Fouse and Hodgson, 1987), and carbon tetrachloride (CCl4) (Klingensmith et al., 1983; Klingensmith and Mehendale, 1982). Our group has reported that chlordecone can potentiate hepatic fibrosis in mice with CCl4-induced chronic liver

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; CD, Chlordecone; ConA, concanavalin A; MHV3, murine hepatitis virus type 3

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injury (Tabet et al., 2016) and that chlordecone can be quantified and localized *in situ* in liver by MALDI imaging (Lagarrigue et al., 2014). Here, we investigated the effect of chlordecone in acute hepatitis, caused by an hepatotropic/neurotropic viral agent, and auto-immune liver diseases. Two mouse models of acute hepatitis were developed, the first one by co-exposure of mice to chlordecone and Concanavalin A (Con A) as an inducer of auto-immune hepatitis (Tiegs et al., 1992) and the second by co-exposure to chlordecone and mouse hepatitis virus type 3 (MHV3) virus, which induces viral acute hepatitis and which is also neurotropic (Martin et al., 1994).

2. Materials and methods

2.1. Animals and experimental protocols

Eight-week-old male C57Bl/6 mice were purchased from Janvier labs (Le Genest-sur-Isle, France) and reared under specific pathogen-free (SPF) conditions in the local animal house, in accordance with French laws and institutional guidelines for the handling and care of experimental animals (accreditation of M. Samson #3596). The mice were divided in a random manner into several groups according to the need of the studies. Those treated with chlordecone (Santa Cruz Biotechnology) were given daily doses of 5 mg/kg diluted in olive oil (0.625 g/L diluted in 200 µL of olive oil). Control mice were fed olive oil alone (200 µL). For the chlordecone-Con A co-exposure model, mice were given a daily gavage of chlordecone or oil for 10 days. They were then intraperitoneally injected with 12 mg/kg Con A (Sigma C2010) or PBS and euthanized 10 h later (Suppl. Fig. 1A). For the chlordecone-MHV3 co-exposure model, mice were maintained in individually ventilated cages (Forma Scientific, 1 Marietta, OH) in the BSL3 local animal facility. Mice received daily gavage with 5 mg/kg chlordecone diluted in olive oil (0.625 g/L diluted in 200 µL of olive oil) or oil (200 µL) for 10 days. For *in vivo* viral inoculation, the pathogenic L2-MHV3 strain were injected by intraperitoneal (i.p.) route at 10^3 50% tissue culture infective dose (TCID50) per animal as described previously (Bleau et al., 2016) or with PBS as control. Mouse blood was collected 48 and 72 h after infection at the time of euthanasia. In the survival study, mice were weighed daily, morning and evening, and their body temperature measured. In all studies, mice were regularly observed and weighed and their blood was collected at the time of euthanasia. The liver was removed and a total lobe of this organ was fixed for 24 h in 4% paraformaldehyde and embedded in paraffin or frozen in liquid nitrogen in the presence of the cryoprotectant isopentane. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined according to IFCC primary reference procedures, with an Olympus AU2700 Autoanalyser® (Olympus Optical Optical Co. Ltd., Tokyo, Japan).

2.2. Histopathology

For the assessment of liver injury, 4-µm-thick sections of paraffin-embedded liver were cut, placed on microscope slides, and stained with hematoxylin and eosin (H&E). Slides were scanned with a Nanozoomer NDP and images acquired using NDP viewer software. The measurement of necrotic area presented in mouse liver was carried out using the ImageJ software. All necrotic area were selected and measured as a percentage of the total area of the liver section presented on the slide. The results showed for the measurement of necrotic area represents the livers of all the mice used in the study.

2.3. RNA isolation and RT-qPCR

We homogenized tissue samples weighing approximately 100 mg and extracted total RNA in Trizol reagent (Invitrogen). The first-strand cDNA was produced with SuperScriptTM II Reverse Transcriptase (Invitrogen). The cDNA was then amplified, with the GAPDH housekeeping gene as a control. Quantitative PCR was performed with the fluorescent dye SYBR Green and the double strand-specific SYBR® Green system (Applied Biosystems), using an ABI 7900 H T Prism sequence analyzer (Applied Biosystems). Total cDNA (30 ng) was used as a template for amplification, with the specific primer pair used at a final concentration of 300 nM. Each measurement was performed in triplicate. We determined 18S mRNA levels as a control, and the level of expression of each gene studied is expressed relative to that of the 18S gene. The primers used are presented below:

Gene	Forward	Reverse
18S	5'CGCCGCTAGAGGTGAAATTC3'	5'ATGCACATCAATGTGGAGGA3'
N-MHV-3	5'TGGAAGGTCTGCACCTGCTA3'	5'TTTGGCCCACGGGATTG3'
IL-8	5'AGAGTCCCGCTGACCAAGAG3'	5'CACTGACAGCGCAGCTCATT3'
IL-6	5'CTGATGCTGGTGACAACCAC3'	5'CAGAATTGCCATTGCACAAC3'
IFN-γ	5'AGGTCAACAACCCACAGGTC3'	5'ATCAGCAGCGACTCCTTTTC3'
TNF-α	5'TAGCTCCCAGAAAAGCAAGC3'	5'TTTTCTGGAGGGAGATGTGG3'

2.4. Immune cell isolation and flow cytometry assay

After euthanasia, mouse livers were perfused through the hepatic portal vein with 2 mL PBS. Immune cells were prepared from liver crushed on a 70 µm filter. Liver immune cells were isolated after sedimentation and cell fractionation on a 35% Percoll layer. For each organ, red blood cells were lysed with the ammonium-chloride-potassium (ACK) buffer. To exclude dead cells from analysis, cell suspensions were labeled for 30 min with LIVE/DEAD fixable yellow stain (Life technologies, L34959). Cells were also pre-incubated with an anti-CD16/32 antibody (dilution 1/100) (BD Pharmingen) to block non-specific binding, before the incubation with the appropriate fluorochrome-conjugated antibodies (BD Pharmingen, eBioscience): anti-CD3-V500 (clone 500A2) (dilution 1/50), anti-TCRβ-V450 (clone H57-597) (dilution 1/50), anti-CD69-FITC (clone H1.2F3) (dilution 1/100), anti-CD19-APC (clone 1D3) (dilution 1/100), anti-NK1.1-PerCP-Cy-5.5 (clone PK136) (dilution 1/200), anti-CD4-PE-Cy7 (clone RM4-5) (dilution 1/50), and anti-CD8-APC-Cy7 (clone 53-6.7) (dilution 1/100). The stained cells were analyzed on an Aria II flow cytometer using BD FACSDiva software and the data were processed using CXP software. Dead cells and doublets were excluded based on the forward and side scatter and LIVE/DEAD staining. Immune cell phenotyping was: LT4 : $CD3^+/TCRV\beta^+/NK1.1^-/CD4^+$; LT8: $CD3^+/TCRV\beta^+/NK1.1^-/CD8^+$; NKT: $CD3^+/TCRV\beta^+/NK1.1^+$; NK: $CD3^-/NK1.1^+$; LB : $CD19^+/CD3^-$; macrophages: $CD11b^+/GR1^{int}$; and neutrophils: $CD11b^+/GR1^{high}$. CD69 was used to study lymphoid cell activation.

2.5. Statistical analysis

Kruskal–Wallis one-way analysis of variance (ANOVA) was performed and mean differences between experimental groups were