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Allergens present in *Molicola horridus* (Cestoda, Trypanorhyncha), a cosmopolitan fish parasite

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Abbreviations: CLE, crude larval extract; CME, crude fish muscle extract

Background: Fish-borne parasitic allergens can induce allergic manifestations even when the parasitized fish is well cooked. The cestode *Molicola horridus* is a muscle parasite of teleost fish, such as the swordfish.

Aims of the study: To determine the allergenic potential of the components present in the crude larval extract (CLE) of *M. horridus*.

Methods: Two mouse models were exposed to the CLE: adult BALB/c mice that were intraperitoneally immunized and newborn BALB/c mice that were orally exposed. Specific antibody levels in serum and faeces were measured by ELISA. The cellular immune response was determined by proliferation assay of splenocytes from sensitized mice. The protein profile of CLE was analysed by SDS-PAGE and western blot.

Results: In adult mice, specific IgG and IgA were detected in sera and faeces, whereas specific IgE were detected in sera only. In newborn mice, specific IgG were detected in sera and a low level of IgA was detected in faeces. SDS-PAGE revealed the CLE protein profile, with most of the proteins running from 15 to 50 kDa. Specific IgG recognized mainly the 26 and 75 kDa proteins and a molecular complex below 100 kDa by immunoblot. Specific IgE recognized the same 26 kDa protein as IgG did, and, with less intensity, another protein at 30kDa. Splenocytes from CLE-immunised mice proliferated when stimulated with CLE in a dose-dependent manner.

Conclusions: The crude larval extract from *M. horridus* has allergenic molecules which can represent a serious risk for fish consumers.

Key words: food allergy, fish parasite, *Molicola horridus*, oral exposure, intraperitoneal exposure

The consumption of raw or undercooked parasitized sea-food can represent a serious public-health risk (1). Fish parasites can induce several pathologies, whose clinical manifestations can range from mild symptoms (e.g., diarrhoea) to severe symptoms (e.g., acute abdomen). In the already sensitized host, even the consumption of well-cooked parasitized fish can induce allergic manifestations, since some of the allergens involved in such reactions are thermo-stabile (2,3).

To date, most attention has been focused on nematode worms of the genus *Anisakis* (3,4), yet there are numerous sea-food-borne parasitic allergens (1). For example, the allergenic capacity has been demonstrated for protozoa belonging to the Phylum Myxospora (5) and for the Trypanorhyncha cestode *Gymnorhynchus gigas*, which are very often found in the muscles of bramids (*Brama raji*) (6). However, nothing is known about the allergenic capacity of another cosmopolitan Trypanorhyncha cestode, *Molicola horridus*, which parasitizes the liver and muscles of teleost fish. *Molicola horridu*s has been frequently detected in swordfish (*Xiphias gladius*) and short moonfish (*Mola mola*) in the Mediterranean Sea and along the coasts of Australia, Canada, India, Japan, and New Zealand. This cestode has also been documented in the spiral valve of the elasmobranche shortfin mako (*Isurus oxyrinchus*), captured along the coasts of Japan and Southern California (7).

Like other parasites infecting the somatic muscles of fish, the larval stage (known as “plerocercoid”) of *M. horridus* develops into cysts that are easily visible to the naked eye, decreasing the edible portion and resulting in economic loss. Moreover, since there is no effective method for detecting the cysts without destroying the tissue, the parasitized fish can reach the consumer. This is particularly important in the industry of tinned and homogenized baby foods. In the European Union, about 35,000

tonnes of swordfish are consumed yearly (www.flmnh.ufl.edu/fish/Gallery/Descript/Swordfish/Swordfish.html).

The objective of the present study was to determine the allergenic capacity of the crude larval extract (CLE) from the plerocercoids of *M. horridus* and its ability to induce sensitization when administered orally. To this end, two mouse models were exposed to the CLE antigens: adult BALB/c mice, exposed intraperitoneally (i.p.), and newborn BALB/c mice, exposed per os.

Materials and methods

Parasites and soluble extracts

The plerocercoids of the parasite *M. horridus* were manually collected from the skeletal muscles of swordfish purchased at fish-markets in Italy, using forceps and scissors. The larvae, approximately 14 cm long, were localised in canalicols parallel to the backbone and spread in all muscles except the superficial lateral muscles. The scolex is a large structure with 4 bothridia and 4 contractile and retractable proboscids which are covered by numerous rows of hooks. *Molicola horridus* presents a sinusoidal chain of small hooks on the lateral surface of the proboscid. The distal part of the proboscid is completely covered by long hooks.

After the morphological identification, the plerocercoids were separated from fish tissues and ridden of their membranes by rinsing them several times in sterile 0.1M phosphate buffered saline (PBS) pH 7.3 and then in PBS supplemented with 5% penicillin and 5% streptomycin. The plerocercoids were then homogenized on ice in a PTFE-on-glass tissue homogenizer for 1 min, with a 1-min pause, 20 times; they were then sonicated on ice for 30 sec with 30 sec pauses, 6 times. The suspension was

centrifuged at 60,000 *g* at +4°C for 30 min. The supernatant was filtered using a 0.22 µm filter (MillexGV; Millipore, Molshe, France). The CLE protein content was estimated using Bradford's protein assay (8). The same protocol was used to prepare a crude muscle extract (CME) which was used as control to treat per os the control mouse group and as antigen for control serological assays.

Animal model, parenteral immunization and oral administration

Ten-week old (adult) and one-week-old (newborn) BALB/c mice (purchased from Charles River Laboratories; Milan, Italy) were housed in the Animal Care Unit of the *Istituto Superiore di Sanità* and treated according to the European Directive 8/609 EEC.

Before each immunization, a suspension was prepared by mixing the CLE (50µg/mouse) with the commercial alum gel suspension ($Al(OH)_3$, 13mg/mL, 200µL/mouse, Sigma-Aldrich, Milan, Italy). In the first experiment, one group of adult BALB/c mice (n=7) was inoculated i.p. with 50 µg of CLE in $Al(OH)_3$, on days 0 and 21. The control mouse group (n=7) received i.p. $Al(OH)_3$ at the same times. Blood samples were taken by tail bleeding at 0, 7, 14, 21, 28 and 35 days after the first immunization. Individual sera were stored at -20°C until analysis.

In the second experiment, one group of one-week old BALB/c mice (n=6) received four weekly doses of 200µg/50µl of CLE by a bucco-gastric tube. The control group (n=3) received the same dose of CME at the same times. Blood samples were taken by tail bleeding at 0, 7, 14, 21, and 25 days after the first treatment. Individual sera were stored at -20°C until analysis.

Faecal samples were collected from all animals at weekly intervals; the samples were weighed and dissolved in 1.0 ml of PBS containing 0.1% sodium azide per 100 mg of faecal material. They were then vortexed for 5-10 min. After centrifugation, supernatants were collected and stored at -70°C.

Detection of specific antibodies in serum samplesand faecal pellets

Total specific IgG, IgE, IgG1 and IgG2a to *M. horridus* CLE were measured in serum samples and specific IgG1 and IgA in faecal samples by an ELISA. Microtiter plates (Nunc-Immuno Plate Polysorp™, Brand Products, Denmark) were coated with 5µg/mL of CLE in carbonate/bicarbonate buffer, pH 9.6, and incubated at 37°C for 90 min. Serum samples from treated and control mice were diluted in PBS with 0.5%w/v BSA and 0.05% v/v Tween-20; faecal samples were diluted as described above. Plates were washed three times between each incubation step with 0.05% PBS-Tween-20. Non-specific binding sites were blocked with 200 µL per well of PBS, 1% w/v BSA at room temperature (RT) for 90 min. Faecal contents and sera were then added to the wells (diluted 1:20 for IgE and 1:100 for IgG, IgG1 or IgG2a and IgA) and incubated overnight at 4°C. The experiment was performed in duplicate. The pre-immune faecal content and the serum from each individual mouse were used as negative controls. After washing, bound antibodies were detected by adding a biotin conjugated rat monoclonal anti-mouse IgG, IgA, IgE, IgG1 and IgG2a (BD Biosciences Pharmingen, San Diego, CA, USA) at RT for 5 h followed by 2.5 mg/mL avidin peroxidase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The secondary antibodies were used at 1:500 dilution. The peroxidase substrate (3,3',5,5'-tetramethylbenzidine, TMB, Kierkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added and the optical density was

determined by an ELISA reader at 450nm. ELISA was performed for each isotype using CME as antigens, following the same protocol as above.

Recognition of potentially allergenic proteins by western blot

The CLE was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 4%-12% gradient gel. For the immunoblot, proteins were separated in a 10% resolving gel and transferred to nitrocellulose membranes. The non-specific binding of antibodies was blocked by incubating the membranes with 2% FCS in TNT (Tris/HCl pH 8.0 with 0.05% Tween-20) at RT for 1 h. After blocking, the membranes were incubated overnight at RT with a 1:50 (for IgE) or 1:1,000 (for IgG) mouse immune sera or with mouse pre-immune sera as control, diluted in a blocking buffer. After washing, the nitrocellulose was exposed to a 1:1,000 biotin conjugated rat monoclonal anti-mouse IgE (BD PharMingen, San Jose, CA, USA) for 3 h at RT, followed by treatment with 2.5 µg/mL of avidin peroxidase or 1/5,000 peroxidase labelled goat anti-mouse IgG (Biorad, Hercules, CA, USA) at RT for 1 h, all diluted in blocking buffer. The IgE/antigen interaction was revealed with the ECL system (Amersham Biosciences, UK). The peroxidase substrate (3,3'-diaminobenzidine, Sigma, Saint Louis, MO, USA) was then added to develop the IgG/antigen interaction.

Specific stimulation of splenocytes with CLE

After the last blood sampling, the adult mice used in the first experiment were sacrificed by vertebral dislocation, and spleens were harvested under sterile conditions. After spleen disruption by a syringe and erythrocyte lysis, splenocytes were

resuspended in a complete medium [RPMI-1640 containing 10% FBS (Hyclone Lab. Inc., Logan, UT, USA), 25mM HEPES, 2mM L-glutamine, 100U/mLpenicillin, 100µg/mL streptomycin, 1mM sodium pyruvate, 5.5 x 10^{-5} M 2-mercaptoethanol, and 0.1 mM non-essential amino acids, all from Hyclone, at a final concentration of 1 x 10^{6} cells/mL (flat-bottom 96-well plates, Costar Corporation, Cambridge, MA)] for the proliferation. The cell cultures were stimulated with two different doses of CLE (1.0 and 10 µg/mL) in 5%CO_2 at 37°C for 5 days. Negative control wells contained unstimulated cells, whereas positive control wells contained 1.0µg/mL of Concanavalin A. Lymphocyte proliferation was measured by ^{3}H-thymidine incorporation in the presence of 0.5µCi/well [^{3}H]-thymidine (Amersham Life Science, Buckinghamshire, UK) after 12 h of culture. The data were expressed as a stimulation index (SI) (i.e., counts per minute of CLE-stimulated cells divided by counts per minute of unstimulated cells).

Statistical analysis

The Student's t test was used to analyze the data. A *P* value of <0.05 was considered as statistically significant.

Results

Specific antibodies in serum samples and faecal pellets

The intraperitoneal exposure of adult BALB/c mice to CLE (first experiment) induced specific IgG (mainly IgG1) in serum from day 7, and the IgG level progressively increased over time after antigen administration. The highest response was obtained on the last day of observation (i.e., 35 days after the first exposure to CLE

antigens. Specific IgE to CLE was detected in mouse sera 7 days after the last immunization (Fig. 1a, 1b). Specific IgG1 and IgA were detected in faeces 35 days after the first immunization (Fig 1c). No specific antibody response was detected in either the serum or faeces of the control mice (Fig. 1a, 1b, 1c). Moreover, sera and faecal material did not react to CME.

In the second experiment, the oral exposure to CLE of one-week old BALB/c mice did not induce detectable specific IgE in serum, whereas specific IgG was detected 25 days after the first administration (Fig. 2a). In faeces, a low level of IgA but not IgG was detected beginning on day 14 (Fig. 2b). No specific antibody response was detected in either the serum or faeces of control mice orally exposed to CME (data not shown).

Recognition of potentially allergenic proteins by western blot

The protein profile of CLE is shown in Figure 3. Most of the proteins run from 15kDa to 50 kDa; the sharpest bands are at 26 and 30 kDa. Specific IgG induced in mice by i.p. inocula of CLE antigens recognized the 26 and 75 kDa proteins as well as a molecular complex below 100 kDa by immunoblot. Other proteins with an apparent molecular weight of 24, 35 and 50 kDa were also recognized, though the bands were less sharp (Fig. 3 panel B). Specific IgE recognized the same 26 kDa protein as IgG did, and another less sharp band at 30kDa (Fig. 3, panel B). No reactivity was observed when blotted proteins were incubated with pooled mouse pre-immune sera (Fig. 3, panel B).

CLE specific stimulation of splenocytes

Splenocytes from CLE-immunised mice proliferated when stimulated with CLE, in a dose-dependent manner (Fig. 4). No difference in proliferation was found when the

antigenic preparation was boiled at 100°C for 1 hour and used at 10µg/mL as stimulant (data not shown). Splenocytes from all immunized and control mice responded to ConcanavalinA.

Discussion

We assessed the allergenic capacity of the CLE of *M. horridus* following oral and parenteral administration in BALB/c mice, mainly as a function of the humoral response. The high IgE and IgG (mainly IgG1) responses in the i.p. immunized mice (Fig. 1) indicate the allergenic nature of CLE. BALB/c mice have proven to be an appropriate model for identifying and characterizing protein allergens; in fact, serological responses induced in BALB/c mice after the i.p administration of potential allergens appear to distinguish between allergenic proteins and other proteins which, despite being immunogenic, fail to provoke an IgE response. (9). In fact, several studies have shown the value of BALB/c mice in oral sensitization protocols, both when using an adjuvant and not (10-12). In the present study, only IgG, and not IgE, was detected in the sera of BALB/c mice orally exposed to CLE (Fig. 2). Although oral administration may more closely reflect human exposure, the ability of BALB/c mice to mount an IgE response after oral sensitization may vary. In fact, oral administration may not possess the sensitivity or reliability to provide an initial assessment of allergenic potential (13). Thuswe cannot exclude the possibility thatoral exposure to CLE induces sensitization.

In the gut, the IgA response to CLE was detected in both oral and i.p. models of exposure to the *M. horridus* antigens (Figs. 1 and 2), and these findings are compatible with the hypothesis that allergen-specific IgA plays a role in the allergic inflammation (14).

We identified two IgE-binding proteins in CLE by immunoblotting: one at 26kDa, which was cross reactive with IgG, and another one at 30kDa (Fig. 3, panel B). Regarding fish parasites, to the best of our knowledge, only the allergens of *A. simplex* have been studied and characterized, yet only in part (3); however, for other fish parasites, the proteic pattern is unknown, although their ability to trigger the production of anaphylactic-type antibodies has been reported (5, 6). A 24 kDa collagenase has been identified in *G. gigas*, a parasite that is closely related to *M. horridus*, given that they are both cestodes of the order Trypanorhyncha (7). The *G. gigas* 24 kDa collagenase is a target of both local (intestinal) and systemic humoral responses in mice sensitized by a single oral dose of *G. gigas* and it elicits considerable changes in the rat ileum contractility (15). The 24 kDa IgG-binding protein from the CLE of *M. horridus* could be a related protein of the 24 kDa collagenase from *G. gigas*; however, it does not seem to be a major constituent, nor does it appear to be a target of IgE response.

The finding that CLE induced splenocyte proliferation in sensitized mice in a dose-response manner (Fig. 4) and that proliferation was maintained after heat treatment is consistent with the importance of T cells in inducing an IgE response. In conclusion, the present results clearly show the allergenic capacity of components present in CLE from *M. horridus,* stressing the risk for the consumer, also considering that this parasite could act as a “hidden”food allergen.

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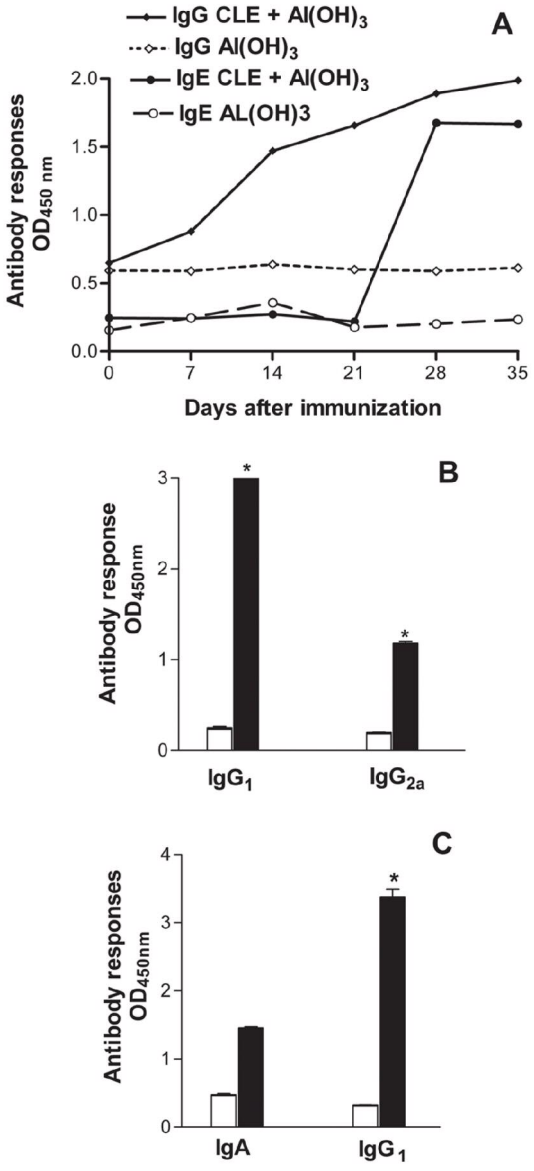
Figure 1: Humoral immune response to crude larval extract (CLE) from *Mollicola horridus* in serum and faeces from $Al(OH)_3$ or CLE + $Al(OH)_3$ intraperitoneal sensitized BALB/c mice. Data are representative of two experiments. Results are expressed as mean ± SEM of individual mouse sera (n=7) (*P < 0.05). (A) Kinetic of specific IgG and IgE responses in serum at 0, 7, 14, 21, 28 and 35 days after the first immunization. (B) Specific IgG1 and IgG2a responses in serum 35 days after the first immunization. Panel C, specific IgA and IgG1 responses in faeces 35 days after the first immunization [black bars, CLE+$Al(OH)_3$; open bars, $Al(OH)_3$]

Figure 2. Kinetic of the humoral immune response to crude larval extract (CLE) from *Mollicola horridus* in serum and faeces of BALB/c mice orally exposed to CLE or to the crude extract from fish muscles (CME) at 0, 7, 14, 21, and 28 days after the first administration. Data are representative of two experiments. Results are expressed as mean ± SEM of individual mouse sera (n=6). (A) Specific IgG and IgE responses in sera. (B) Specific IgA response in faeces.

Figure 3. (A) 4%-12% SDS-PAGE of crude larval extract (CLE) from *Molicola horridus.* After running, proteins were visualized with Coomassie blue. MW, molecular weight marker in kDa. (B) Western blot showing IgG (lane 2) and IgE (lane 3)-binding activity to CLE of pooled sera from CLE intraperitoneally sensitized mice 35 days after the first immunization; proteins from CLE were first separated in a 10% SDS-PAGE; lane 1, mouse pre-immune serum; lane 4, molecular weight marker in kDa.

Figure 4. Splenocyte proliferation from BALB/c mice intraperitoneally immunized with $Al(OH)_3$ or CLE, and stimulated in vitro with Concanavalin A (white bars), with 1µg/mL CLE (grey bars), or 10 µg/mL CLE (black bars). Datafrom five mice, are expressed as a mean stimulation index ± SEM. Data are representative of two experiments.



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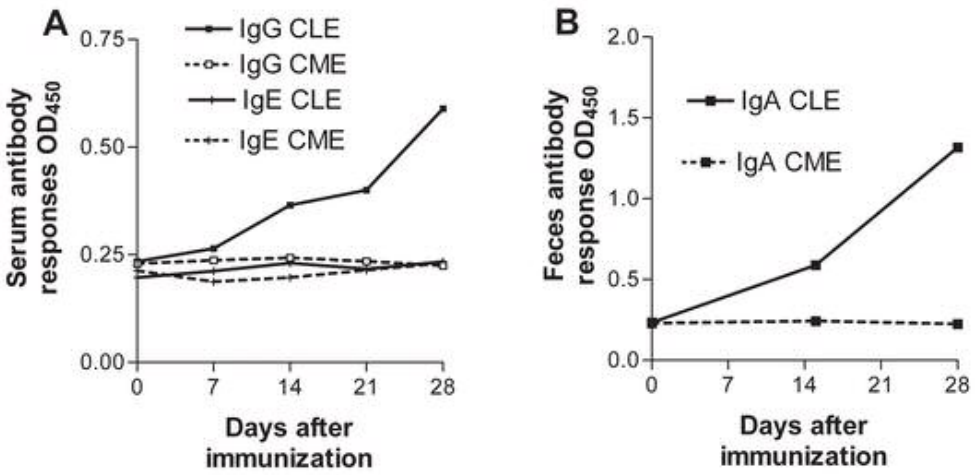

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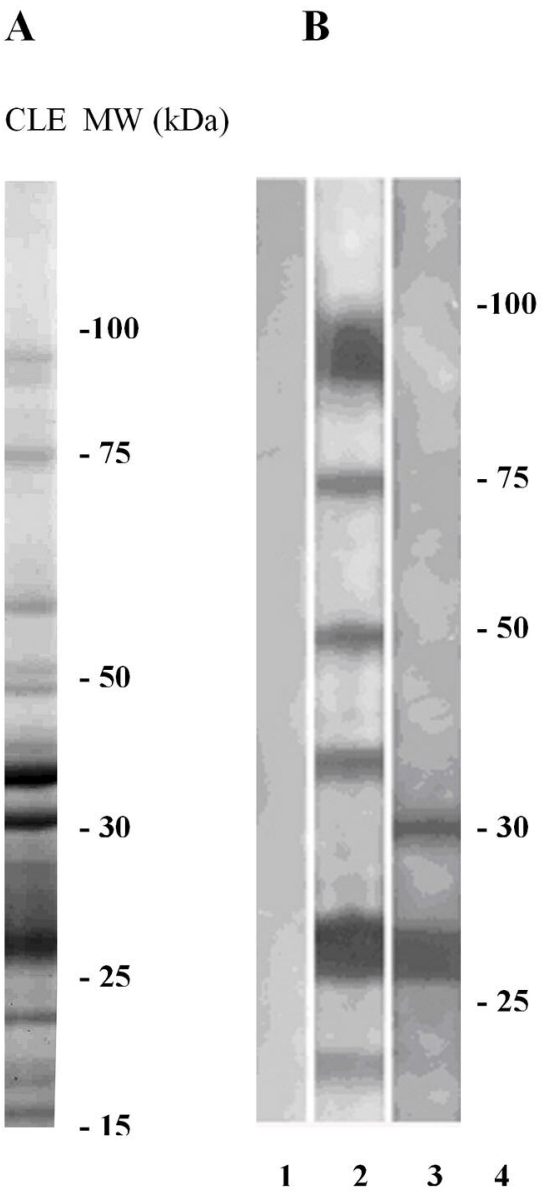

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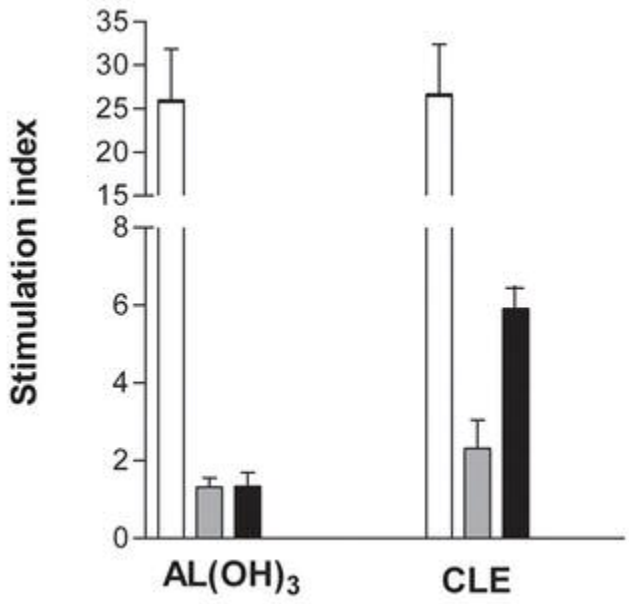

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