1 **Aluminium hydroxide potentiates a protective Th1 biased immune response**
2 **against Polio virus that allows for dose sparing in mice and rats.**

3 Lars Vibe Andreasen², Lasse Bøllehuus Hansen¹, Peter Andersen¹, Else Marie Agger¹, Jes
4 Dietrich^{1*}

6 ¹Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark.

7 ²Department of Vaccine Development, Statens Serum Institut, Copenhagen, Denmark

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13 ***CORRESPONDING AUTHOR:**

14 Jes Dietrich,

15 Department of Infectious Disease Immunology, Statens Serum Institut,

16 Artillerivej 5, DK-2300 Copenhagen S, Denmark

17 Fax: +45 32 68 30 35

18 Email:jdi@ssi.dk

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23 **Abstract**

24 ***Background.***

25 The development of new low cost Inactivated Polio virus based vaccines (IPV) is a high priority
26 and will be essential for the complete eradication of polio. Since the aluminium hydroxide adjuvant
27 is widely used in humans we tested this adjuvant with IPV in two models. Our objective was
28 twofold; to examine the IPV dose sparing effect of aluminium hydroxide and how the adjuvant
29 effect of aluminium hydroxide affected the immunity induced by IPV.

30 ***Methods.***

31 Mice and rats were immunized with IPV formulated with Aluminium hydroxide and subjected to
32 immunological analyses and serum polio virus neutralization titer determination.

33 ***Results.***

34 Addition of aluminium hydroxide to IPV led to a ten times dose sparing effect compared to IPV
35 alone, measured by virus neutralization titers in serum. Aluminium hydroxide changed the kinetics
36 of the response against IPV leading to a faster and stronger response, which due to IPV induced
37 immune dominance was characterized as a strong Th1-biased cellular/humoral immune response.

38 ***Conclusions.***

39 The IPV-aluminium hydroxide formulation constitutes a promising vaccine capable of generating
40 strong Th1 immunity against infection with all three serotypes. A phase I/II clinical study was
41 recently initiated.

42

42

43 **Introduction**

44 Two types of vaccines exist against polio; Inactivated Poliovirus Vaccine (IPV) and live Oral
45 Poliovirus Vaccine (trivalent, bivalent or monovalent). OPV with attenuated Sabin strains of
46 poliovirus types 1, 2 and 3, has been the vaccine of choice for polio eradication in most countries
47 because it induces both systemic and intestinal immunity, can immunize or boost immunity of close
48 contacts through secondary spread, and is inexpensive and easy to administer. However, one
49 challenge with OPV is that on rare occasions OPV can cause vaccine-associated paralytic
50 poliomyelitis (VAPP) and/or revert to a neurovirulent form of poliovirus which is believed to be as
51 transmissible and virulent as wild polioviruses [1-3]. Therefore, steps have been taken to remove
52 OPV as a vaccine against Polio and in a recent WHO position paper it is recommended that
53 countries that today only use OPV should add at least one IPV vaccination to the schedule [4].
54 While there are many benefits of using IPV as the vaccine of choice rather than OPV, purchase
55 costs for IPV is too high for many countries which consequently may result in inadequate supplies
56 of IPV for the afflicted countries. One way to reduce the cost of a vaccine is to use adjuvants [5]. In
57 the field of pandemic influenza vaccines the use of adjuvants has permitted dose reduction,
58 increased the availability of the vaccine and reduced its cost [6-10]. Therefore, it has been
59 speculated that an adjuvanted vaccine formulation of IPV would reduce cost and increase the
60 number of available IPV doses worldwide. In support of this, it was recently shown in animal
61 models that the potency of Salk IPV and Sabin IPV vaccines could be increased with the adjuvants
62 aluminium hydroxide or CpG [11-13]. Moreover, we recently showed that significant IPV dose
63 sparing could be achieved with CAF01, a novel adjuvant composed of cationic liposomes DDA
64 (dimethyldioctadecylammonium) stabilized with the synthetic immunomodulator TDB (trehalose
65 6,6'-dibehenate) [14].

66 Compared to all other adjuvants, aluminium hydroxide is of particular interest as it is the most
67 widely used adjuvant in humans. Aluminium adjuvants have been used in practical vaccination for
68 more than 60 years and are generally recognized as safe and efficient [15].

69 In this study we therefore tested the ability of the aluminium hydroxide adjuvant to increase the
70 protective immunity against an infection with polio virus. Humoral immunity is believed to be the
71 principal effector mechanism to combat Polio. This was shown as early as in a trial performed in the
72 1950s where the effect of passive transfer of immunoglobulin was examined [16]. Thus, apart from
73 constituting a safe vaccine [17], IPV is also able to induce a significant protective immunity on its
74 own. Therefore, although the primary goal for this study was to achieve dose sparing with
75 aluminium hydroxide, an important secondary objective was to examine how the adjuvant effect of
76 aluminium hydroxide, traditionally thought to be an inducer of Th2 immunity, would combine with
77 the ability of IPV to induce its own protective immunity.

78

78

79 **Methods**

80 **Ethics Statement**

81 Experiments were conducted in accordance with the regulations set forward by the Danish Ministry
82 of Justice and animal protection committees by Danish Animal Experiments Inspectorate Permit
83 2004-561-868 (of January 7, 2004) and in compliance with European Community Directive 86/609
84 and the U.S. Association for Laboratory Animal Care recommendations for the care and use of
85 laboratory animals. The experiments were approved by the Statens Serum Institut IACUC.

86

87 **Animal handling**

88 Studies were performed with 6- to 8-wk-old female CB6F1 C57BL/6xBALB/c mice from Harlan,
89 Scandinavia or Wistar/HAN rats from Charles River laboratories, Germany. Animals were housed
90 in appropriate animal facilities at Statens Serum Institut.

91

92 **Mono- and trivalent IPV**

93 The IPV vaccines in this study used commercial GMP grade materials manufactured at SSI. The
94 trivalent IPV vaccines were formulated in the ratio 40:8:32 for polio virus type 1 (Brunhilde strain),
95 type 2 (MEF-1) and type 3 (Saukett), respectively, throughout the study. The trivalent IPV stock
96 (IPV TP) applied for the vaccines was batch TPA 2011-01, determined at the time of use to contain
97 327:70:279 DU/mL of type 1, 2 and 3, respectively.

98

99 **IPV-aluminium hydroxide formulation (IPV-AI)**

100 The vaccines containing aluminium hydroxide were formulated from a 3% Alhydrogel (Brenntag
101 Nordic, Denmark) suspension. The aluminium suspension was diluted with buffers/M199 to the

102 appropriate aluminium concentration prior to add-mixing 1:1 v/v with an IPV solution. The dose of
103 aluminium hydroxide was 100 ug/dose in both mice and rats. Physical/chemical analysis of the
104 IPV-AI formulation showed that IPV was tightly associated with aluminium hydroxide and
105 maintained its D-unit activity in the vaccine formulation despite the presence of the adjuvant.
106 Furthermore, the DU activity of the vaccine did not show a major decline after 8 months at cold
107 storage (Supplemental figure 1).

108

109 **Immunization**

110 Mice were immunized two or three times at two-week intervals intramuscularly (IM) with IPV or
111 IPV formulated with aluminium hydroxide. The doses used in the experiments are indicated in the
112 figures.

113

114 **Lymphocyte cultures**

115 Lymphocytes were cultured and purified as described previously [18].

116

117 **Cytokine ELISA and Multiplex cytokine assay**

118 A sandwich ELISA was used to determine the concentration of IFN- γ in culture supernatants as
119 previously described [18]. The Th1/Th2 cytokine multiplex assay (Meso Scale Discovery,
120 Gaithersburg, MD) were performed according to the manufacturer's instructions.

121

122 **DU ELISA**

123 D-antigen was quantified using a sandwich ELISA, as previously described [14].

124

125 **Microneutralization assay performed at SSI**

126 Neutralizing antibody titers to the three poliovirus serotypes 1, 2 and 3 were measured using
127 Brunhilde, MEF-1, and Saukett virus stocks on Vero cells, as previously described [14].
128

129 **Microneutralization assay performed at CDC**

130 Samples were tested in triplicate using a standard microneutralization assay for antibodies to
131 poliovirus types 1, 2, and 3 according to established protocols at the Global Polio Specialized
132 Laboratory, Centers for Disease Control and Prevention (CDC). Briefly, 80-100 CCID₅₀ of each
133 poliovirus serotype and two-fold serial dilutions of serum were combined and pre-incubated at 35°C
134 for 3 hours before addition of HEp-2(C) cells. After incubation for 5 days at 35°C and 5% CO₂,
135 plates were stained with crystal violet and cell viability measured by optical density in a
136 spectrophotometer. Each specimen was run in triplicate, with parallel specimens from one study
137 subject tested in the same assay run, and the neutralization titers estimated by the Spearman-Kärber
138 method and reported as the reciprocal of the calculated 50% endpoint. Each run contained multiple
139 replicates of a reference antiserum pool starting at a 1:32 dilution to monitor performance variation.
140 A serum sample was considered positive if antibodies were present at $\geq 1:8$ dilution. Specimens with
141 antibody titers $< 1:8$ were considered seronegative and specimens with titers $> 1:8$ were considered
142 seropositive.

143

144 **Measurements of antibody titers**

145 Animals were bled for the collection of serum 2 weeks following the vaccination 1-3, or as
146 indicated in the figure, and antibody titers were determined as described previously [14].
147

148 **Statistical methods**

149 A significant difference in immune responses measured by ELISA, or a difference in neutralization
150 titers, was evaluated using a one-way ANOVA, and Tukey's Multiple Comparison Test for multiple
151 comparisons. A value of $p < 0.05$ was considered significant. Prism version 5 software (GraphPad)
152 was used for analysis and the standard settings of $\alpha=5\%$ and $\beta=20\%$ were used.

153

154

154

155 **Results**

156 **Aluminium hydroxide induced dose sparing**

157

158 We first investigated to which degree dose sparing of IPV could be achieved in the mouse model by
159 applying aluminium hydroxide to IPV. Mice were immunized with 20 DU IPV or 2 DU IPV with or
160 without aluminium hydroxide (indicated dose units in the all experiments correspond to Polio virus
161 type-1 D antigen units). The mice received two immunizations spaced by two weeks and
162 neutralizing antibody titers were determined in the sera at week 2 post the last immunization.

163 The results showed that aluminium hydroxide induced an increase in neutralization titers (Fig. 1).

164 The increase in titers against Type 1 and 3 were significant, whereas the difference between type 2
165 titers in the IPV 2DU and IPV 2DU + aluminium hydroxide groups were not significant (as Type 2
166 titers were too high to allow for an adjuvant dose sparing effect). Neutralization titers to type 1
167 increased from 3.3 +/- 0.9 (Log₂ transformed titers) in the IPV 2DU group to 7.7 +/- 1.5 in the IPV
168 2DU + aluminium hydroxide group. Neutralization titers in the IPV20 DU group were 5.1 +/- 1.8
169 demonstrating a 10x dose sparing effect of aluminium hydroxide regarding Type 1 titers. The same
170 patterns was seen with Type 3 ((IPV 2DU: 6.7 +/-2.1, IPV-AI 2DU: 9.0 +/- 1.1, IPV 20 DU: 9.2 +/-
171 1.3). In conclusion, addition of aluminium hydroxide allowed for a ten times dose sparing of IPV.

172

173 **Aluminium hydroxide induced dose sparing in the rat model**

174 As the rat model is a frequently used model in the polio field we also tested the IPV-AI formulation
175 in this model. The effect of aluminium hydroxide was tested with an IPV dose of 1.6 DU after two
176 and three vaccinations. Three weeks after two vaccinations, 0.1 mg aluminium hydroxide induced a
177 10 times dose sparing. Thus, formulated with 1.6 DU IPV, neutralization titers (Type 1: $13.45 \pm$
178 0.15 , Type 2: 13.35 ± 0.34 , Type 3: 12.90 ± 0.70) measured by the assay developed at SSI [14] at
179 least equaled those observed in the IPV 16DU non-adjuvanted group (Type 1: 13.31 ± 0.84 , Type 2:
180 12.81 ± 1.16 , Type 3: 12.37 ± 1.39) (Fig. 2A). Moreover, these results were confirmed using
181 another Polio virus neutralization assay developed at CDC (Fig. 2B). The same pattern was also
182 apparent after three vaccinations despite the increased neutralization titers (Fig. 2C and D).
183 Furthermore, the titers after two vaccinations with the IPV-AI 1.6DU formulation were not
184 significantly different compared to titers observed after three vaccinations with the non-adjuvanted
185 IPV formulation. This was observed using both neutralization assays (Fig. 2E and F). Thus, also in
186 the rat model a x10 dose sparing can be achieved by using the adjuvant aluminium hydroxide
187 formulated with IPV.

188

189 **Aluminium hydroxide induced an increase in IgG binding titers**

190 As IPV possesses the ability to mount a protective immune response by itself, it was important to
191 examine how IPV induced immunity was affected by the adjuvant. Two weeks after the second
192 (Fig. 3A, B, and D) or third (Fig. 3C) vaccination sera from individual mice were analyzed for IPV
193 specific antibody titers measuring both the different IPV specific IgG isotypes, as well as the total
194 IgG level, against the three virus serotypes. The binding titers in sera from mice vaccinated with
195 IPV-AI 2DU were higher than the IPV 2DU group against all the serotypes, and at least as high as
196 the IPV 20DU group (Fig. 3A). Aluminium hydroxide led to increased antibody titers for all the

197 IgG isotypes, and in particular we observed a strong IgG2a, IgG2b, and IgG2c response after two
198 and three vaccinations (Fig. 3B and C). This is in contrast to what is normally seen with a
199 recombinant protein formulated with aluminium hydroxide, where IgG1 usually represent the
200 dominant isotype (supplemental figure 2 A and B). Increased IgG titers in the aluminium hydroxide
201 group was also observed 8 and 11 weeks following the second vaccination, demonstrating that it
202 was not merely a transient increase in binding titers (Fig. 3D).

203

204 **Aluminium hydroxide increase the cellular response**

205 To further characterize the IPV-AI formulation we also analyzed the cellular response against IPV.
206 Cellular immunity towards polio virus has received less attention due to compelling evidence that
207 humoral immunity is the most important effector mechanism against polio. However, there are
208 numerous examples showing that cellular immunity, besides being an effector mechanism in itself,
209 can be important for humoral immunity [19-25], even humoral immunity against polio virus [26,
210 27]. PBMCs were obtained from mice vaccinated with 2 DU IPV alone or IPV 2DU + aluminium
211 hydroxide two and five weeks following the booster vaccination. Non-vaccinated mice were
212 included as control. Thereafter, the cells were stimulated in vitro with IPV for 72 hours and the
213 supernatant was collected for Multiplex cytokine analysis. Two weeks post vaccination 2, the
214 highest response in the non-adjuvanted IPV 2DU group was observed with IL-2 (525 ± 165 pg/ml).
215 In contrast, in the IPV-AI 2DU group we observed a significant increase in secretion of cytokines
216 IFN- γ , IL-17, IL-5, IL-10, IL-2 and TNF- α (Fig 4A). In particular, at week 2 post vaccination
217 aluminium hydroxide led to strong induction of IFN- γ (2661 ± 534 pg/ml versus 153 ± 86 pg/ml
218 in the IPV 2DU group) and IL-10 (4294 ± 1993 pg/ml versus 232 ± 138 in the IPV 2DU group)
219 (Fig. 4A).

220 At week 5 post vaccination, in the IPV alone groups we observed an increased secretion of
221 cytokines IFN- γ (to 2536 +/- 443 pg/ml) and IL-2 (928 +/- 35 pg/ml) (Fig 4B). In the IPV 2DU +
222 aluminium hydroxide group we observed an increase in IFN- γ (to 6382 +/- 914 pg/ml) and IL-2 (to
223 1310 +/- 688 pg/ml) ($p < 0.05$), but a significant decrease in secretion of IL-17, IL-5 and IL-10,
224 compared to week 2 post vaccination (Fig 4B and C). Thus, at week 5 the secretion of cytokines
225 was dominated by IFN- γ and IL-2 in both the adjuvanted and non-adjuvanted group. IL-13 was also
226 measured at week 5 post vaccination but was not increased significantly by aluminium hydroxide
227 (402 +/- 273 pg/ml in the IPV 2DU group versus 576 +/- 307 in the IPV-Al 2DU group) (data not
228 shown). Finally, also in the rat model did aluminium hydroxide lead to elevated levels of Th1
229 cytokines (IFN γ and TNF α), in contrast to Th2 cytokines (Fig. 4D).

230 Taken together, aluminium hydroxide induced a faster and stronger cellular response with a Th1
231 profile similar to the profile induced by non-adjuvanted IPV (Fig. 4B and C).

232

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236

237 **Discussion.**

238 In the present study we show that addition of aluminium hydroxide to IPV led to a ten times dose
239 sparing effect in mice and rats, compared to IPV alone, as well as a faster and stronger Th1
240 dominated response.

241

242 *Achieving dose sparing with aluminium hydroxide in two animal models.*

243 It is well known that a prerequisite for the implementation of IPV worldwide as a replacement for
244 OPV is the availability of affordable IPV in low and middle-income countries. Lower cost options
245 being pursued include reducing the number of doses and the amount of antigen per dose [11-13,
246 28]. As antigens, both Salk and Sabin IPV are being considered in combination with aluminium
247 hydroxide. A recent phase I study showed no vaccine-related serious adverse events following a
248 booster administration with one dose of Sabin-IPV adjuvanted with aluminum hydroxide [29].
249 However, a dose sparing effect previously observed in mice [11] could not be transferred to humans
250 with the experimental setup used in the experiment [29].

251 We were able to achieve a ten times dose sparing effect measured by the virus neutralization titers
252 in the blood in both mice and rats (Fig. 1 and 2). Importantly, in the rat model the results also
253 showed that two vaccinations with IPV 1.6DU + aluminium hydroxide gave neutralization titers
254 that equaled those induced by 3 vaccinations with IPV 1.6DU (Fig 2 E and F). Thus, addition of the
255 adjuvant aluminium hydroxide led to a significant dose sparing effect, or allowed for a vaccine
256 schedule with one less dose. The increased aluminium hydroxide induced neutralization titer was
257 mediated by IgG as only a small amount of IgA was induced (data not shown). Regarding induction
258 of IgA, which is believed to be important to reduce virus shedding, we recently showed with
259 CAF01 that an intradermal administration can increase intestinal IgA [14]. Alternatively, a

260 supplemental dose of IPV-AI administered to children previously exposed to OPV should also
261 increase intestinal IgA [30-34]. Our results, using Salk inactivated poliovirus vaccine, are in
262 agreement with a recent study that showed that it is possible to increase the potency of Salk and
263 Sabin inactivated poliovirus vaccines by using aluminium hydroxide or CpG [11-13], and also in
264 other vaccines did aluminium hydroxide facilitate dose sparing [6, 7]. The amount of dose sparing
265 induced by aluminium hydroxide correlated with increased vaccine induced immunity and was
266 found to be comparable with that induced by the adjuvant, CAF01[14].

267

268 *The aluminium hydroxide induced immune response against IPV*

269 Although the cellular immune response is not considered a direct correlate of protection for polio
270 vaccines it may play an indirect role by supporting a memory B cell response [35, 36]. Our analysis
271 of anti-IPV T cell immunity revealed that aluminium hydroxide induced both a faster and stronger
272 response against IPV. However, the cytokine profile of the aluminium hydroxide group changed
273 significantly from week 2 to week 5, into a profile that resembled the profile induced by IPV alone,
274 a profile dominated by IFN- γ secretion (Fig. 4). Thus, IPV as an antigen can determine the type of
275 cellular (and humoral) immunity induced against it, even in the presence of aluminium hydroxide.
276 Similar observations were made with the CAF01 adjuvant in a recent study using the mouse model
277 [14]. As Polio virus is a positive stranded RNA virus it can most probably trigger Toll-like receptor
278 7 and 8 [37] which has been shown to lead to a Th1 polarization of the immune response
279 characterized by elevated expression of Th1 cytokines and a switch from an IgG1 towards a IgG2a
280 isotype in the mouse [38-41]. Interestingly, triggering of Toll receptor 7/8 by adding Resiquimod
281 (known to bind TLR 7/8) to Aluminium hydroxide also leads to a Th1 dominated immune response
282 (Supplementary figure 2). The observed T cell profile presented in our study is in agreement with a
283 previous study showing the same cytokine profile following immunization with Sabin IPV +/-

284 aluminium hydroxide [26]. In the IPV-AI formulation it is probably beneficial that IPV is the
285 immune-dominating factor since, as discussed above, there is evidence that Th2 cell-associated
286 mediators (and IL4 in particular) may in fact exert a negative impact on immune protection and
287 drive immunopathology during infection with a number of viruses [42-46].

288

289 **Concluding remarks**

290 In conclusion, our results show that both in the mouse and rat model the aluminium hydroxide
291 adjuvant allowed for a ten times IPV dose sparing measured by the polio virus neutralization titers
292 and the IPV IgG binding titers. Regarding the immune response, our data indicated that rather than
293 determine the type of immune response induced by the IPV-AI formulation, the role of aluminium
294 hydroxide is to potentiate an immunity already determined by IPV, the latter most probably being
295 an advantage as this immunity has already been shown to be highly protective in humans. How the
296 findings in the present study will translate to humans will be revealed in the near future as the IPV-
297 aluminium hydroxide formulation presented here is presently being tested in a phase I/II clinical
298 trial.

299

300 **Figure legends.**

301 **FIGURE 1.** Aluminium hydroxide induced dose sparing measured by virus neutralization titers

302 Virus neutralization antibody titers from mice (n = 8/group) 14 days following the second
303 immunization with IPV (with or without adjuvant). Neutralizing antibody titers against poliovirus
304 serotype 1-3 are shown. The individual titers for each mouse are plotted and the bar represents the
305 mean neutralizing antibody titer. SEM of estimated $\log_2$ values are shown, *, $p < 0.05$, as indicated in
306 the graph using one-way ANOVA and Tukeys post-test for multiple comparisons. Indicated dosage
307 units in the experiments correspond to Type 1 D-antigen units. Results are representative of 3
308 experiments.

309

310 **FIGURE 2.** Aluminium hydroxide induced dose sparing in the rat model

311 **A-D.** Virus neutralization antibody titers from rats (n = 10/group) 14 days following the second (A
312 and B) or the third (C and D) immunization with inactivated polio vaccine (with or without
313 adjuvant). Titers in A, C and E were determined with the SSI (Statens Serum Institute) assay. Titers
314 in B, D, and F were determined with the CDC assay (developed at Center for Disease Control,
315 Atlanta). Neutralizing antibody titers against poliovirus serotype 1-3 are shown. The individual
316 titers for each rat are plotted and the bar represents the mean neutralizing antibody titer. SEM of
317 estimated $\log_{10}$ values from N=4 rats per group *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ are indicated in
318 the graph using one-way ANOVA and Tukey's post-test for multiple comparisons. Indicated dosage
319 units in the experiments correspond to Type 1 D-antigen units. **E and F.** Virus neutralization
320 antibody titers from rats (n = 10/group) 14 days following the second or the third immunization
321 with inactivated polio vaccine (with or without adjuvant). Neutralizing antibody titers against
322 poliovirus serotype 1-3 are shown. The titers are from the SSI assay and the CDC assay. The

individual titers for each rat are plotted and the bar represents the mean neutralizing antibody titer. SEM of estimated $\log_{10}$ values from N=10 rats per group *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ are indicated in the graph using one-way ANOVA and Tukey's post-test for multiple comparisons. Indicated dosage units in the experiments correspond to Type 1 D-antigen units.

FIGURE 3. Aluminium hydroxide increase the Antibody binding titers

A. The serum IgG against TP or the individual virus serotypes was measured by indirect ELISA using trivalent IPV or the MPs as the antigen. The Ab titer was measured by the reaction of a series of 10-fold dilution of sera with the antigen. The Ab titration from the sera of 4 mice are shown for the vaccine groups indicated. **B and C.** The serum IgG1, IgG2a, IgG2b and IgG2c against TP was measured by indirect ELISA using trivalent IPV as the antigen (N=6-8) post vaccination 2 (B) or 3 (C). **D.** Serum IgG against TP 8 and 11 weeks post the second vaccination in the vaccination groups indicated (N=6). Indicated dosage units in the experiments correspond to Type 1 D-antigen units.

FIGURE 4. Aluminium hydroxide induced cellular immunity in mice and rats.

A and B. Mice (N=8) were vaccinated twice with IPV 2DU (white bars) or IPV 2DU + aluminium hydroxide (black bars). At week 2 or week 5 post vaccination 2, PBMCs were stimulated with IPV for 72 hours and secretion of the indicated cytokines was analyzed by MSD. The cytokines are indicated in the figure. **C.** Week 5 post vaccination 2 responses. Indicated dosage units in the experiments correspond to Type 1 D-antigen units. **D.** Rats were vaccinated twice with IPV 1.6DU or IPV 1.6DU + aluminium hydroxide, three weeks apart. At week 2 post vaccination 2, PBMCs were stimulated with IPV for 72 hours and secretion of the indicated cytokines was analyzed by MSD.

347

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359

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498 ¹Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark.

499 ²Department of Vaccine Development, Statens Serum Institut, Copenhagen, Denmark

500

501 *Corresponding author

502 Jes Dietrich,

503 Department of Infectious Disease Immunology, Statens Serum Institut,

504 Artillerivej 5, DK-2300 Copenhagen S, Denmark

505 Fax: +45 32 68 30 35.

506 Email: jdi@ssi.dk

Figure1

Figure2

Figure3

Figure 4