Vaccine 29 (2011) 3596–3599

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Pharmacology and toxicology of an oral tablet whole cells inactivated cholera vaccine in Sprague Dawley rats

Yulieé López[a], Juan Francisco Infante[a], Sergio Sifontes[a], Daiyana Díaz[a], Viviana Pérez[a], Gemma Año[a], Tamara Hernández[a], Sonsire Fernández[a], Jorge Luis Castaño[b], Bárbara Cedré[a], Reynaldo Oliva[a], Luis García[a], Rosa L. Solís[a], Arturo Talavera[a,*]

[a] Finlay Institute, Center for Research-Production of Vaccines, 27th Ave. No. 19805, La Lisa, Ciudad de la Habana 11600, Cuba
[b] MEDSOL Laboratories, 23rd Ave and 266th St., Playa, Ciudad de la Habana, Cuba

ARTICLE INFO

Article history:
Received 1 September 2010
Received in revised form 18 February 2011
Accepted 22 February 2011
Available online 6 March 2011

Keywords:
Cholera
Inactivated vaccine
Tablets
Pharmacology
Toxicity
Sprague Dawley rats

ABSTRACT

Here we further investigate the pharmacological and toxicological properties of a cholera vaccine based on inactivated whole cells presented in either enteric coated (COA) or uncoated (U/C) tablet formulation from *Vibrio cholerae* C7258 strain. Tablets were dispersed in 2 mL drinking water and administered orally to Sprague Dawley rats distributed in five groups (I COA7, II U/C7 immunized at 0, 7, 69 days and III COA14, IV U/C14 immunized at 0, 14, 69 days and V control group). Serum vibriocidal antibody response was measured after the administration of two doses with an interval of 7–14 days. To further investigate the toxicological aspects a third dose was applied 10 weeks after the initial one. Animals were observed daily and water and food consumption was measured every other day. Periodic blood extractions were performed for hematology, biochemistry, and the titer of serum vibriocidal antibodies was determined. Anatomopathological analysis was performed at days 3 or 14 after the third dose. Results from clinical observations, as well as from water and food consumption and body weigh indicated no toxicity of the vaccine product. Meanwhile, no biological differences were found among different groups in hematological, hemo-chemistry, and anatomopathological studies. Moreover, enteric coated and uncoated tablets against human cholera were found to induce an immune response in rats.

1. Introduction

Human Cholera is one of the three most serious diseases in the World Health Organization (WHO) Compelling Report. Thousands of cholera cases are reported annually worldwide, but the Word Health Organization estimates that only a fraction of such cases are informed [1]. Cholera is a disease predominantly affecting poor countries with deficient health care systems and scarce means for drinking water and sewage treatments [2]. Thus, vaccination stands as the best short-term cost-benefit choice.

Three types of *Vibrio cholera* vaccines are being currently investigated around the globe: inactivated whole cells vaccines, live genetically attenuated vaccines and subunit vaccines. Typically, the administration of all oral cholera vaccines required a gastric juice protection [3], but the Vietnamese experience suggests that this is not necessary [4]. Therefore, for future clinical development of the tablet vaccines it is important to evaluate the preclinical toxicity of uncoated and coated tablets as final pharmaceutical forms.

In general, preclinical testing is a prerequisite for moving a candidate vaccine from the laboratory to clinical testing in humans, which includes product characterization, proof of concept and immunogenicity studies, and safety testing in animals [5]. A WHO Guide Line did not recommend general safety (innocuity) for final lot control of inactivated oral cholera vaccine presented in liquid form [6]. However, the solid presentation in tablets could introduce some concerns because it has been never applied to human and their toxicity or it has been evaluated in animal models.

The most relevant biomodel for the preclinical trials is the adult Sprague Dawley rat, in which a single oral dose of the attenuated cholera vaccine resulted immunogenic (scheme proposed for humans), allowing the assessment of the intrinsic toxicity of vaccine components as well as the potential toxicity associated to the immune response [7].

It has been previously shown that rats respond immunologically to the oral administration of *Vibrio cholerae* whole cells and to the subunits of such microorganism [8]. Likewise, previous trials using the corresponding candidate vaccine active pharmaceutical ingredient have proved that in a two dose scheme, with an interval of 14 days, rats respond with vibriocidal antibody titers comparable to those obtained with a single or two dose of the living

* Corresponding author. Tel.: +53 7 2716911; fax: +53 7 2086075.
E-mail address: atalavera@finlay.edu.cu (A. Talavera).

0264-410X/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2011.02.074

attenuated candidate vaccine (Talavera et al., unpublished results). Thus, Sprague Dawley rats constitute a potential relevant model to evaluate the intrinsic toxicity as well as the toxicity associated to the immune response of vaccine products against cholera [8].

The objective of this study was to determine the potential toxicity of oral whole cells inactivated cholera vaccine in uncoated and coated tablets by pharmacological and toxicological preclinical tests.

2. Materials and methods

2.1. Evaluated products

Tablets of whole cells inactivated cholera vaccine were produced under Good Manufacture Practice and maintained at room temperature. The active pharmaceutical ingredient was a suspension of *V. cholerae* C7258 inactivated cells (7 mg dry weight per core). The cores were obtained as described previously [9]. A part of core batch was kept uncoated (U/C), while the rest of the batch was processed to be coated (COA) with Kollicoat® MAE-100P polymer (BASF) at a core temperature of $42 \pm 2\,^{\circ}C$, following manufacturer's instructions.

2.2. Animal model

Females Sprague Dawley rats (CENPALAB, Havana, Cuba) with body weight of 160–180 g and males with 220–250 g body weight were used in the trials.

Animals were placed in type 4 polycarbonate boxes (floor area: 1800 cm^2) (Tecniplast, Italy), containing autoclaved sugar cane shredded bagasse as lodging. Animals followed a 10 h light–14 h darkness cycle and were fed *ad libitum* with specialized fodder for rats (CENPALAB, Havana, Cuba). Drinking water was supplied *ad libitum*. The rooms were kept at 20–25 °C and relative humidity of 60–65%.

2.3. Immunization

Each dose consisted of a COA or U/C tablet dispersed into 2 mL of drinking water and was delivered orally via a cannula for rats. Tablet dispersions remained in agitation during the whole time of administration. No anesthetic or sedation means was used.

After an initial period of adaptation to housing and feeding conditions, rats were distributed in five groups, conformed by 16 females and 16 males rats each. Groups I (COA7) and II (U/C7) were immunized at 0, 7 and 69 days, rats of groups III (COA14) and IV (U/C14) were immunized at 0, 14 and 69 days and rats of group V (control) were not immunized. All the experimental procedures were performed blind including the group distribution product and samples results. The data were registered in a primary forms and once the study ended, the codes were opened to obtain a full interpretation of the results.

2.4. Clinical studies

Animals were weighed at the beginning and at the end of the adaptation period, every seven days during the trials and at the day of sacrifice in order to obtain their weight after fasting. Consumed water volume and food weight per box were registered every other day.

The clinical follow-up was carried out daily, paying special attention to the manifestation of piloerection, prostration, involuntary movements, ataxia, salivation, lacrimation, excitation or depression, incoordination and diarrhea.

2.5. Serological studies

Serology was performed weekly up to third dose, and 3 or 14 days after the last immunization. 100 μL of blood without anticoagulant were collected and processed for vibriocidal test. The positive reactors were considered as vibriocidal titers ≥ 20 as described [10].

2.6. Sacrifice of animals

Sacrifice was executed by chloroform anesthesia and bleeding the animals through the femoral artery. Eight animals per sex of each experimental group were sacrificed at days 3 and 14 after the third immunization for hematological, serologic, hemochemical and anatomopathological studies.

Blood samples of 1.5 mL were collected for vibriocidal titers and biochemical studies in vials without anticoagulant, centrifuged and sera preserved at $-20\,^{\circ}C$ until the moment to perform the analysis.

2.7. Hematological and hemochemical determinations

Total erythrocytes and leukocytes, and differential leukocyte (neutrophils and lymphocytes) count were done.

The biochemical study included the determination of total bilirubin, direct bilirubin, creatinine, urea, uric acid, triglycerides, amino transferase aspartate (TGO-ASAT) and alanine amino transferase (TGP-ALAT) were measured by using diagnostic kits (CENTIS Diagnostics, Havana).

2.8. Anatomopathological studies

They included necropsy and histopathology study of the following organs: tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, mesenteric ganglia, liver, spleen, kidneys, parotid gland, lungs, heart, and thymus. Heart, right and left lungs (R/L), kidneys (R/L), thymus, liver and spleen were weighed to determine their weight relative to the body weight.

2.9. Statistical analysis

Data were stored in Microsoft and were processed using the Statgraphic Plus 5.0software. In all cases, statistical significance was set to $p < 0.05$.

A multiple comparison analysis, including the assessment of data standardization and variance equality, was performed for the continuous variables (body weight, water and food consumption). Data groups meeting the aforementioned parameters were processed by ANOVA, and those others which did not, were processed using Kruskall–Wallis tests. A hypothesis test with binomial distribution was used for the comparison of proportions (splenitis frequency and vibriocidal reactors).

3. Results and discussion

To evaluate a satisfactory safety margin of the product, we took into account the weight and allometric relations between man and rat, and used the proposed vaccine dose of one tablet via oral for clinical use in humans.

The immunization scheme of oral cholera vaccines based on inactivated whole cells comprises the application of two doses [7]. Previous experiments using the active pharmaceutical ingredient of the vaccine under study evidenced the necessity of a second dose to induce a significative response of serum vibriocidal antibodies in rats. Therefore, we applied two doses to simulate the scheme conceived for humans and to determine if the seven days schedule

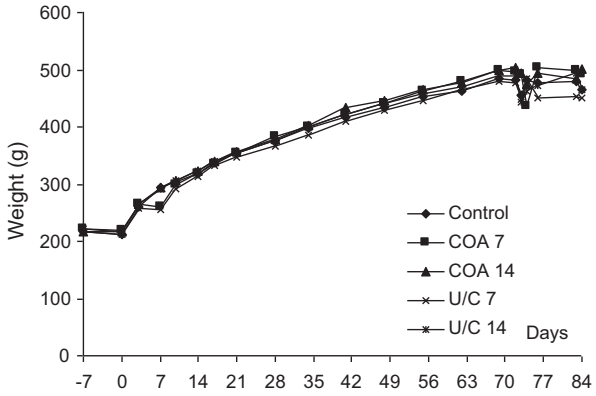

Fig. 1. Average weight of control rats (male) or immunized via oral with coated or uncoated tablets ($N = 16$ up to day 72, then $N = 4$). Control: Non-immunized rats. COA 7: Rats immunized at days 0, 7 and 69 with dispersed coated tablets. COA 14: Rats immunized at days 0, 14 and 69 with dispersed coated tablets. U/C 7: Rats immunized at days 0, 7 and 69 with dispersed uncoated tablets. U/C 14: Rats immunized at days 0, 14 and 69 with dispersed uncoated tablets.

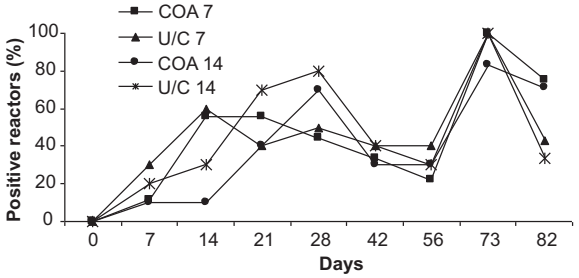

Fig. 2. Female positive reactors (vibriocidal titers ≥ 20) during the assay. The rats of the control group did not show positive reactors. COA 7: Rats immunized at days 0, 7 and 69 with dispersed coated tablets. COA 14: Rats immunized at days 0, 14 and 69 with dispersed coated tablets. U/C 7: Rats immunized at days 0, 7 and 69 with dispersed uncoated tablets. U/C 14: Rats immunized at days 0, 14 and 69 with dispersed uncoated tablets.

is not inferior to the one of 14 days. In addition, once the path of the immune response was evaluated, an extra dose was applied to conduct toxicological studies.

We found that all groups behaved homogeneously to the different treatments, showing similar weight average (Fig. 1). The statistical analysis did not show significant differences among the groups according to treatments of a same sex and experimental day in agreement with other toxicological vaccine reports [11]. At the day 7th the immunized groups showed less weight than others, but these result might have been due to the 24 h fasting to which the rats had been submitted. Water consumption average was significantly different ($p < 0.05$) for each sex among the different experimental groups, being higher in males that in females, as reported [10]. Average food consumption average was not significantly different between experimental groups ($p < 0.05$), but was higher in males than in females as reported previously [12,13].

No clinical symptoms were observed during the trial in any group.

We performed a serological study to assess the relevance of this biomodel for the assay by determining whether animals responded to the immunogen. Positive reactors began to appear after the 7th day of the first immunization in all immunized groups. These increased further at the 14th day in the in the groups who received the second dose at 7th day, and at the 21th day, in those who received the second dose at 14th day. The reactor percentage was maintained at high values between 21 and 28 days, decreased up to day 56. Reactor percentages were attained the highest values 4 days after the third immunization and tended to decrease 14 days later.

The evaluation of male sera yielded similar behavior, but with lower values than those obtained in the female group (data do not shown), which is in agreement with the smaller response of vibriocidal antibodies of males with respect to females [14].

The vibriocidal titers in serum samples from rats immunized with either coated or uncoated dispersed tablets showed that these animals are capable of responding to the immunogen under evaluation. It is also important to notice that all groups presented an increment of positive reactor percentages 4 days after the application of the third dose, that is, 69 days after initiation of the immunization schedule, and 56 or 63 days after the second dose. On the other hand, no significant differences were found between the responses to the two immunization schedules with coated or uncoated tablets (see Fig. 2).

The results of the hematological analysis (counts of erythrocytes, leukocytes, lymphocytes and neutrophils) yielded no statistically significant differences between treatment groups, including the control group. In all groups the average erythrocyte count was in the range from 8×10^6 to 12×10^6 cell/mL, the leukocyte count was between 8.3×10^3 and 14×10^3 cell/mL, the lymphocyte count was between 64×10^3 and 82×10^3 cell/mL and the neutrophil count ranged between 18×10^3 and 36×10^3 cell/mL.

All biochemical determinations were in normal range and did not show significative statistical differences among groups, including the control group (total proteins, 59.6–66.5 mg/mL; direct bilirubin, 0.975–1.09 μM/L; total bilirubin, 2.41–3.13 μM/L; TGP, 24.75–32.86 U/L; urea, 5.13–5.9 μM/L; TGO, 65.12–75.87 U/L; triglyceride, 0.8–1.18 g/L and creatinine, 70–86 μM/L. Average values and ranges recorded for all these variables were, in general, similar to reference figures reported in the scientific literature [8,11]. Our results differed from previous reports in animals suffering of adverse events during toxicological studies [15,16,17].

Averages of organ relative weight (organ weight/live weight × 100) were not significantly different between rats from different groups and corresponded to the normal values reported by Pritchett and Coming [18].

Table 1 summarizes the anatomopathological alterations found in the rats sacrificed 3 or 14 days after the last immunization. Given that lesions were observed with very low frequency and in all experimental groups, including the control group, they had not toxicological significance.

Histopathological analysis yielded that no significant differences between groups.

We found that the most frequent alterations were related to the immune system, and in particular, to the immune system associated to the digestive mucosa. Vibriocidal antibody analysis revealed that the digestive mucosa of affected animals resulted immunogenic, in accordance to previous reports from Kkunishi et al. and Didia and Dapper [19,20]. However, such alterations could also obey to the wide exposure of the digestive tract to the environment, as similar changes had been previously described in healthy rats raised in a conventional environment [21,22]. In any case, the immune hyperplasia observed here was not significantly different between treatment and control groups.

On the other hand, epidemiological studies in endemic areas indicate that natural infection with *V. cholerae* induces a long-lasting protective immunity characterized by high levels of antibacterial IgAs, which is translated into an effective mucosal immune response. At serum level, it is also characterized by IgG anti LPS titers, antitoxins and vibriocidal antibodies [23].

Studies in volunteers experimentally inoculated with virulent strains or challenged after immunization have demonstrated that there is a strong relation between protection and vibriocidal antibodies in serum. This suggests that IgG could also play an important role at the mucosa. However, the non-invasive nature of *V. cholerae*

Table 1
Anatomopathological alteration in rats sacrificed after 3 or 14 days after last immunization.

Groups	U/C 7				U/C 14				COA 7				COA 14				Control			
Sacrifice times	3 day		14 day		3 day		14 day		3 day		14 day		3 day		14 day		3 day		14 day	
Anatomopathological alterations	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Immune system hyperplasia associated to small intestine	3	4	6	4	5	6	6	5	5	3	4	6	4	5	6	6	2	3	5	5
Light hyperplastic splenitis	0	1	0	2	0	0	0	1	1	1	0	0	1	2	1	1	0	0	0	1
Medium hyperplastic splenitis	0	0	2	1	2	1	1	1	0	0	0	2	0	0	2	2	0	0	1	1
Grave hyperplastic splenitis	0	0	1	0	0	0	2	0	0	0	1	1	0	0	0	0	0	0	0	1
Paracortical follicles hyperplasia in lymphatic ganglions	1	2	0	0	0	1	0	0	3	3	0	0	2	2	0	0	1	0	0	0
Mononuclear perivascular infiltrate in pulmonary veins	2	2	1	0	0	1	0	0	2	2	2	3	1	1	1	0	2	2	1	2
Round cells among the glands of the small intestine mucous	1	0	1	1	0	0	0	2	0	0	0	2	0	0	1	2	0	0	0	2
Immune system hyperplasia associated to large intestine	1	2	0	2	3	4	3	1	1	3	1	0	2	2	2	0	1	2	1	3
Secondary follicles in lymphatic ganglions	0	0	0	3	1	2	2	4	0	0	0	3	2	1	2	5	1	2	1	3

Control: Non-inoculated rats. COA 7: Rats inoculated with dispersed coated tablets at days 0, 7 and 69. COA 14: Rats inoculated with dispersed coated tablets at days 0, 14 and 69. U/C 7: Rats inoculated with dispersed uncoated tablets at days 0, 7 and 69. U/C 14: Rats inoculated with dispersed uncoated tablets at days 0, 14 and 69. F: Females. M: Males.

and the fact that the immune system enhancement occurs at Peyer's patches [24] suggest that vibriocidal antibodies in serum are not real mediators of protective immunity, but that they work as indicators of the presence of IgA intestinal secretor antibodies.

In conclusion, the animal model used here is capable of responding to the immunogens presented in either coated or uncoated tablets, applied with two different immunization schedules. Interestingly, sex seems to exert an influence, as females displayed stronger responses than males. Importantly, no deaths or symptoms suggestive of toxicity were reported with either pharmacological presentations or immunization schedules. On the other hand, macroscopic alterations observed consisted in splenitis processes equally affecting all groups. Biochemical and hematological tests did not reveal alterations that could be attributed to the assayed product. Consequently, none of the parameters tested showed biological differences of toxicological value, allowing us to state that this tablet vaccine against human cholera did not result toxic for the animal model used in our assay conditions.

Acknowledgments

We thank Mildrey Fariñas, Adriana Ponce, Bárbara Yolanda Valdés, Jorge Luis Prieto, Eligio A. Sosa and Niurka Rodríguez for technical assistance.

References

[1] Word Health Organization. WHO. Weekly Epidemiological Record, No. 31; 4 August 2006.
[2] Talavera A, Pérez EM. Is cholera disease associated with poverty? J Infect Dev Ctries 2009;3(6):408–11.
[3] Walter RI. Considerations for development of whole cell bacterial vaccines to prevent diarrheal diseases in children in developing countries. Vaccine 2005;23:3369–85.
[4] Anha DD, Canha DG, Lopez AL, Thiema VD, Longc PT, Sona NH, et al. Safety and immunogenicity of a reformulated Vietnamese bivalent killed, whole-cell, oral cholera vaccine in adults. Vaccine 2007;25:1149–55.
[5] Word Health Organization. Nonclinical evaluation. Available from: http://www.who.int/biologicals/areas/vaccines/nonclinical_evaluation/en/index.html.
[6] Word Health Organization. Guidelines for the production and control of inactivated oral cholera vaccines. WHO Technical Report, Series No. 924; 2004.
[7] Proceedings of the workshop on non-clinical safety evaluation of preventive vaccines: recent advances and regulatory considerations, vol. I, Monday, December 2, 2002, Arlington, Virginia. Miller Reporting Co., Inc., 735 8th Street, S.E. Washington, DC 20003-2802, (202) 546–6666.
[8] Sifontes S, Infante JF, López Y, Fariñas M, Claveras A, Sosa E, et al. Toxicity study per repeated dose of the *V. cholerae* attenuated vaccine in Sprague Dawley rats. Arch Med Res 2009;40(7):527–35.
[9] Talavera A, Año G, Pino Y, Castaño J, Uribarri E, Riverón L, et al. Formulation in tablets of a cholera whole cells inactivated vaccine candidate. Vaccine 2006;24:3381–7.
[10] Cedré B, García H, García L, Talavera A. Estandarización del ensayo Vibriocida modificado para la determinación de anticuerpos contra *Vibrio cholerae*. Rev Cubana Med Trop 1999;51(3):56–9.
[11] Infante JF, Sifontes S, Álvarez E, González M, Pérez V, Sosa E, et al. Toxicity study per single dose of vax-SPIRAL vaccine in Sprague Dawley rats. Vacci Monitor 2004;13(2):11–5.
[12] Canadian Council on Animal Care and Use. Laboratory rats. Guide to the care and use of experimental animals, vol. 2. Ottawa, Ontario: Canadian Council on Animal Care and Use; 1993. p. 175–87 [chapter XXI].
[13] Pérez GRM, Montalvo DM, Rodríguez AJC, Calderón MCF, Cruz MJ, Bello GJL. Determination of toxicity in reiterative dose of the arsenical. Oncol Cuban Mag 2001;17(2):111–7.
[14] Sincharoen Kul R, Chaicumpa W, Pongponratn E, Limpanonont J, Tapchaisri P, Chogsa-Nguan M. Localization of *Vibrio Cholerae* 01 in the intestinal tissue. Asia Pac J Allergy Immunol 1993;11(2):155–65.
[15] Abraham P, Kanakasabapathy I, Dian BJ. Propylthiouracil attenuates acetaminophen-induced renal damage in the rat. Nephrology 2005;10:588–93.
[16] Senthilkumar S, Devaki T, Manohar BM, Babu MS. Effect of squalene on cyclophosphamide-induced toxicity. Clin Chem Acta 2006;364:335–42.
[17] Tasduq SA, Mondhe DM, Gupta DK, Baleshwar M, Johri RK. Reversal of fibrogenic events in liver by Emblica officinalis (fruit), an Indian natural drug. Biol Pharm Bull 2005;28:1304–6.
[18] Pritchett KR, Coming BF. Biology and medicine of rats. In: Ruter JD, Suckow MA, editors. Laboratory animal medicine management. Ithaca NY: International Veterinary Information Service; 2004. www.ivis.org; B 2503. 0904 (last updated: 29.11.04).
[19] Kkunishi H, Sudo M, Sumi M, Morii H, Minuchi T, Aimoto T, et al. Pharmacokinetic behavior of Micafungin in rats with carbon tetrachloride-induced acute hepatic failure. Biol Pharm Bull 2005;28(3):556–9.
[20] Didia BC, Dapper DV. Regional variation in the histological appearance of the small and large intestine of the rat following acute and chronic administration of Melo's Conguer Mixtare. West Afr J Med 1999;18(2):110–3.
[21] Infante JF, Sifontes S, Pérez, Bracho G, Hernández T, Zayas C, et al. Immunogenicity assay and local toxicity of *Neisseria meningitidis* cochlear Sprague Dawley rats. Vacci Monitor 2009;18(1):1–7.
[22] Fariñas M, López Y, Infante JF, Oliva R, Sosa E, Sifontes S, et al. Non toxicity for a single dose study and local tolerance of the vaccine against *Neisseria meningitides* serogruop A and C using Sprague Dawley rats. Vacci Monitor 2009;18(2):41–2.
[23] Jertborn M, Svennerholm AM, Holmgrin J, Salina. Breast milk and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J Clin Microbiol 1986;24(2):203–9.
[24] Glass RI, Svennerholm AM, Stoll BJ, Khan MR, Hossain KM, Huq MI, et al. Protection against cholera in breast-fed children by antibodies in breast milk. N Engl J Med 1983:308–92.