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Effects of Buprenorphine on Immunogenicity and Protective Efficacy in the Guinea Pig Keratoconjunctivitis Model (Sereny Test)

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Shigellosis is a disease of global proportions, with an estimated 164.7 million episodes annually throughout the world as well as an estimated 1.1 million associated mortalities in developing countries. Due to increasing incidence, and continued emergence of multi-drug resistant strains, *Shigella* vaccine development is considered a top public health priority. The guinea pig keratoconjunctivitis model, the basis for the Sereny test, remains the most reliable in vivo indicator of virulence of *Shigella* strains and immunogenicity and protective efficacy of *Shigella* vaccine candidates. The model is effective in evaluating the ability of *Shigella* strains to invade the corneal epithelia of guinea pigs and spread to contiguous cells, with the more virulent strains causing ulcerative keratoconjunctivitis. However, analgesia is not routinely used to relieve this painful condition because of potential immunomodulation and confounding of experimental results. The objective of the study reported here was to evaluate use of buprenorphine hydrochloride as an analgesic during the Sereny test. Local and systemic immune responses were measured in guinea pigs given buprenorphine versus those responses in controls. Results of this study suggest that buprenorphine, administered at an analgesic dose of 0.05 mg/kg of body weight twice daily, can be successfully used with the model without significantly affecting immunologic evaluation of *Shigella* vaccine candidates. However, in buprenorphine-treated animals, there was a significant increase in the amount of mucopurulent ocular discharge, requiring frequent cleaning of the affected eyes. Additionally, animals treated with buprenorphine had significant reduction in body weight, in comparison with saline controls.

There exist moral, ethical, and legal mandates to relieve unnecessary pain and distress in animals used in biomedical research (1-5). These mandates present special challenges for investigators conducting research involving non-alleviated pain (5, 6). An example of this type of research is the guinea pig keratoconjunctivitis model, known as the Sereny test (ST). This model is used to characterize the virulence of *Shigella* strains and the immunogenicity and protective efficacy of *Shigella* vaccines (7-9). The ST, which mimics the invasive processes of *Shigella* sp. in the intestinal epithelia of humans and primates, involves introducing live organisms into the eyes of guinea pigs and evaluating the ability of these bacteria to invade corneal epithelia and spread to contiguous cells (10-14). A highly virulent strain will cause marked ulcerative keratoconjunctivitis, whereas a vaccine strain that is not sufficiently attenuated will cause some disease or irritation. Since this correlates well with reactogenicity in humans, a strain such as the latter would be a less than favorable vaccine candidate (9). The importance of these findings has been well-documented in several human trials (15-20). Although several alternative in vitro models are available in *Shigella* vaccine research, they fail to quantitatively measure the in vivo effects of the host's immune response, including development of protective immunity, thereby necessitating use of the guinea pig keratoconjunctivitis model (21-24). The US Food and Drug Administration recognizes the model, and results of the ST, as a reliable indicator of vaccine strain attenuation. These attenuation studies are mandatory prior to phase-I vaccine trials.

Development of *Shigella* vaccines is considered a top public health priority of the World Health Organization and the Department of Defense due to the high incidence of shigellosis in developing countries and in deployed military personnel (25-29). However, use of the ST poses many bioethical concerns; the main one is pain to the guinea pigs. *Interagency Research Animal Committee Principles For the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* states "unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals" (4). The pain of ulcerative keratitis, as is often observed in the guinea pig keratoconjunctivitis model, is typically described by humans who have had this condition as dull, throbbing, and persistently severe (30). Furthermore, the pain threshold in humans appears to be similar to that in animals (31). Use of analgesics, however, can directly or indirectly affect experimental outcomes by altering physiologic and behavioral responses, thereby potentially invalidating the research model (5). Previous concerns regarding use of the guinea pig keratoconjunctivitis model prompted an Institutional Animal Care and Use Committee (IACUC)-sponsored study to evaluate the effects of analgesics on the development of disease in the ST. Results of the study indicated that the opioid buprenorphine hydrochloride could be used in conjunction with the test, without significantly interfering with the disease process (32). Questions were subsequently raised regarding the drug's potential to effect immunologic responses. These ques-

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tions became the basis for the design and implementation of the follow-up study reported here. Uncertainties about the effects of a drug on experimental outcome are often resolved by conducting a pilot study to evaluate the drug's influence in a limited number of animals (5, 6). The principal objective of this IACUC-mandated study was to evaluate the effects of buprenorphine on the immunologic parameters of the ST and to determine the potential for refinement of the guinea pig keratoconjunctivitis model.

Materials and Methods

Animals. Sixty outbred male Hartley guinea pigs (Crl:(HA)BR, VAF/Plus, Charles River Laboratories, Wilmington, Mass.), weighing between 250 and 300 g, were used in two experiments. The guinea pigs were purchased antibody-free to Sendai virus, pneumonia virus of mice, reovirus-3, lymphocytic choriomeningitis virus, *Encephalitozoon cuniculi*, and *Mycoplasma pulmonis*. Animals underwent a 10-day quarantine on arrival, were singly housed in standard polycarbonate cages (Lab Products Inc., Maywood, N.J.) on hardwood bedding (Tek-Fresh, Harlan Teklad, Madison, Wis.), and were provided guinea pig chow (Prolab 5018, Purina Mills, Inc., St. Louis, Mo.) and tap water ad libitum. Environmental conditions included 10 to 15 conditioned fresh air changes/h, a temperature range of 20 to 22°C, relative humidity between 40 and 70%, and a 12-h light/dark cycle with no twilight. All animals were individually identified by use of the Electronic Laboratory Animal Monitoring System (ELAMS, BioMedic Data Systems, Inc., Maywood, N.J.). Studies were approved and funded by the Walter Reed Army Institute of Research IACUC. Guinea pigs were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (2) in a facility that has animal care and use programs accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

In experiment 1, the effects of buprenorphine hydrochloride (Buprenex, Reckitt and Colman Pharmaceuticals Inc., Richmond, Va.) were evaluated following ocular infection with a known virulent *Shigella* strain, on *Shigella* antigen virulence determination, as determined by measuring select immunologic parameters and the ST, using the guinea pig keratoconjunctivitis model. The effect of buprenorphine on the immunologic response was evaluated using an enzyme-linked immunosorbent assay (ELISA) and an enzyme-linked immunospot assay (ELISPOT), both of which have been well-characterized in previous studies (7, 8). Since the O-antigen of the lipopolysaccharide (LPS) molecule is the principal antigen involved in developing protective immunity against shigellosis, *S. flexneri* 2a LPS was the antigen used for analyzing immune responses. The ELISA was used to quantitate the serum IgG and IgA antibody response to *S. flexneri* 2a O-antigen. The ELISPOT assay was used to determine the local immune response to the LPS O-antigen of *Shigella* by measurement of O-antigen-specific antibody-secreting cells (ASC) in the spleen and superficial ventral cervical lymph nodes.

Guinea pigs were randomized into test and control groups, with 14 animals in each group (n = 28). At the beginning of the study (day 0), two guinea pigs were randomly selected from each group (n = 4). From these four guinea pigs, baseline blood samples and tissue specimens were collected for ELISA and the ELISPOT assay. All assays were performed blind, using identical techniques and instrumentation, by a single technician who had no knowledge to which group the selected animals belonged until completion of the study. Blood samples were collected by cardiac puncture, using ketamine (40 mg/kg of body weight) and xylazine (5 mg/kg) for anesthesia. Immediately following blood sample collection, these animals were euthanized, in accordance with the 1993 Report of the AVMA Panel on Euthanasia, with an overdose of pentobarbital, administered intraperitoneally, and their spleen and superficial ventral cervical lymph nodes were immediately harvested for the ELISPOT assay. After these baseline sampling procedures, the remaining guinea pigs from test and control groups (n = 24) were inoculated with 25 µl of *Shigella flexneri* serotype 2a 2457T in phosphate-buffered saline (PBS), approximately 5.0×10^8 colony-forming units (cfu), micropipetted onto the conjunctiva of the right eye.

Virulence of this strain of *Shigella*, including preparation of the inoculum, has been well-characterized in a previous study (7). The animals' eyelids were gently massaged open and shut to ensure even distribution of organisms over the entire ocular surface. The test group received 0.05 mg of buprenorphine/kg administered subcutaneously every 12 h for the duration of the study (33-37). Control group animals received an equal volume of sterile saline for injection, administered in like manner. Guinea pigs were then observed and clinically evaluated twice daily until completion of the study by an experienced observer to whom the identities of test and control groups were unknown. Body weights were recorded daily, and the degree of disease in their eyes, the classic application of the ST, was evaluated and recorded by the observer. The severity rating scale used was as follows: 0 = no disease or mild conjunctivitis, 1 = mild keratoconjunctivitis with conjunctival edema and corneal opacity, but no corneal ulceration, 2 = keratoconjunctivitis with multifocal punctate corneal ulcerations, 3 = fully developed keratoconjunctivitis with diffuse corneal ulceration. Six animals from each group were euthanized on days 7 and 14, and samples were collected as described previously.

In experiment 2, effects of buprenorphine hydrochloride on protective efficacy of live-attenuated vaccine SC602, which has been well-characterized in previous studies (7, 17, 18) against challenge by homologous virulent strain *Shigella flexneri* serotype 2a 2457T, were evaluated. Blind evaluation was performed on the remaining guinea pigs, using the same techniques and procedures as described for experiment 1. The guinea pigs were likewise randomly separated into test and control groups consisting of 14 guinea pigs/group (n = 28). All animals were immunized on day -45 with 25 µl of SC602 vaccine placed into both eyes and received boosters on day -30.

Thirty days following the second immunization (day 0), baseline serum and tissue specimens were obtained for ELISA and the ELISPOT assay from four guinea pigs randomly selected from test and control groups (n = 4). Immunized animals from test and control groups (n = 24) were then challenged with 5.0×10^8 cfu of *Shigella flexneri*, serotype 2a 2457T in PBS, placed into both eyes. Virulence of this *Shigella* strain was validated by inoculating an unvaccinated control group (n = 4) with 5.0×10^8 cfu of the *Shigella flexneri* challenge strain placed into the right eye. These unvaccinated controls were evaluated daily and euthanized immediately on validation of virulence, as detected by development of corneal ulceration (grade 3 on the aforementioned rating scale).

Concurrent with challenge of the immunized test and control

groups (day 0), buprenorphine was administered to the test group animals (n = 12). Sterile saline for injection was administered to the control group (n = 12). Similar to that for experiment 1, each animal was observed twice daily and clinically evaluated, and its body weight was recorded daily. The degree of disease was observed, evaluated, and recorded, using the same Sereny severity rating scale. Serum and tissue specimens were obtained on days 7 and 14, using six animals from the test and control groups (n = 12), by use of the previously described technique.

Statistical analysis. Test animals that were administered buprenorphine concurrent with ocular inoculation or challenge with *Shigella* antigen were compared with control animals that received sterile saline for injection. Serum ELISA titer data (IgA, IgG) and ELISPOT data (ASC from spleen and lymph nodes) were transformed to $\log_{10}$ to achieve more homogeneous variances and to dampen the effects of outliers. Values of "0.0" were assigned a minimal value of "0.1" to enable statistical comparisons. Buprenorphine and saline geometric group means were compared for days 7 and 14, using the two-sample *t*-test for each given day (SAS v.6.12, Cary, N.C.). A value of $P < 0.05$ was considered statistically significant. Ninety-five percent confidence limits for buprenorphine and saline group mean differences were calculated (Microsoft Excel 97). The ST scores, recorded on days 5 after inoculation and after challenge, were analyzed using the Wilcoxon rank sum test to compare medians (SAS v.6.12, Cary, N.C.). Day 5 after inoculation was considered to be the peak day for development of the ST; therefore, all statistical comparisons were based on data obtained on this day. Buprenorphine and saline group mean body weights were compared on day 5, using the two-sample *t*-test, where $P < 0.05$ was considered statistically significant. Ninety-five percent confidence limits for group mean differences also were calculated. An observed finding of increased mucopurulent ocular discharge between test and control groups in experiment 1 was evaluated, using the χ^2-test for association. Where applicable, the guinea pigs' right and left eyes were considered independently for statistical evaluation.

Results

In experiment 1, the effects of buprenorphine on *Shigella* antigen virulence were measured by determination of serum and local antibody response as well as by use of the ST following infection with a known virulent *Shigella* strain. Table 1 shows the mean differences in serum immunoglobulins (IgG, IgA) and O antigen-specific ASC (spleen, superficial ventral cervical lymph nodes) between test and control groups. Ninety-five percent confidence limits and *P*-values for these differences also are indicated. Significant differences were not found between test and control groups. Likewise, results of the ST virulence assay recorded on day 5 after inoculation with *Shigella flexneri* serotype 2a 2457T indicated no significant differences. Although detectable differences were not found for test and control groups ($P = 1.00$), an observational finding of increased mucopurulent ocular discharge was noted in test versus control group animals. Periodically, the observer had to clean the eyes prior to evaluation of the conjunctiva and cornea to prevent adherence of the eyelids and to enable determination of a Sereny severity rating. This finding was determined to be significant ($P < 0.01$). In addition, there was a significant difference in mean body weight between groups five days after the beginning of treatment ($P = 0.02$). All animals treated with saline gained, on average, 18 g, an increase of 4.1%, whereas animals treated with buprenorphine lost an average of 24 g, a decrease of 5.5%. Therefore, the saline-treated animals weighed, on average, 45 g more than did buprenorphine-treated animals after 5 days versus the 3.5-g difference observed between the two groups on day 0.

In experiment 2, the effects of buprenorphine on protective efficacy of *Shigella* live-attenuated vaccine SC602 were evaluated. Table 2 shows the mean differences in serum immunoglobulins (IgG, IgA) and O antigen-specific ASC (spleen, superficial ventral cervical lymph nodes) between test and control groups after immunization and challenge. Ninety-five percent confidence limits for these differences and the corresponding *P*-values also are indicated. Geometric mean differences for splenic IgG and IgA ASC reached significance on day 7 (IgG, $P = 0.02$; IgA, $P = 0.02$). This difference, however, was not detected on day 14. The ST results for immunized test and control groups, five days after challenge with *Shigella flexneri* serotype 2a 2457T, for right and left eyes, were determined not to be significant (right, $P = 0.35$; left, $P = 0.06$).

Similar differences in body weight also were observed. Saline-treated animals gained an average of 30 g after 5 days of treatment, whereas buprenorphine-treated animals lost an average of 24 g; thus, the difference in body weight on day 5 was 66 g versus only 3 g on day 0. This represents a 10.9% difference in body weight between saline-treated and buprenorphine-treated animals ($P = 0.01$).

Discussion

Results of this study indicate that buprenorphine, when used at clinically relevant doses, did not have significant affect on ST severity ratings nor did it interfere with virulence determina-

Table 1. Effects of buprenorphine on virulence evaluation—immunologic response after inoculation with 5 x 10^8 colony-forming units (cfu) of *Shigella flexneri* 2a 2457T

	Day 7						Day 14					
Variable	BUP Mean[a,b]	SAL Mean[a,b]	Diff[c]	LCL	UCL	*P*-value	BUP Mean	SAL Mean	Diff	LCL	UCL	*P*-value
Serum IgG	3.51	2.80	0.71	-0.60	2.01	0.20	3.56	3.35	0.21	-0.40	0.81	0.41
Serum IgA	2.55	1.67	0.88	-0.11	1.88	0.05	2.75	2.55	0.20	-0.40	0.81	0.41
Spleen IgG	0.85	0.81	0.04	-1.41	1.48	0.95	1.21	1.09	0.12	-0.84	1.08	0.75
Spleen IgA	0.58	0.31	0.27	-1.27	1.80	0.67	0.72	1.05	-0.33	-0.96	0.30	0.21
Spleen IgM	0.40	0.51	-0.11	-1.31	1.09	0.82	0.40	0.96	-0.56	-1.51	0.39	0.16
LN IgG	1.47	1.58	-0.11	-1.04	0.83	0.77	1.90	2.05	-0.15	-0.66	0.35	0.45
LN IgA	1.23	0.54	0.69	-0.59	1.97	0.20	1.41	1.64	-0.23	-0.66	0.18	0.18
LN IgM	1.02	1.29	-0.27	-1.45	0.91	0.57	1.44	1.55	-0.11	-0.64	0.41	0.59

[a]Serum IgG and IgA mean values, determined by ELISA, are expressed as geometric mean titers. Optical density read at 405 nm.
[b]Spleen and lymph node IgG, IgA and IgM mean values, determined by ELISPOT, are expressed as geometric mean antibody-secreting cells (ASC) per 10^6 lymphocytes.
[c]Difference between buprenorphine group geometric mean and saline group geometric mean values.
BUP = buprenorphine; SAL = saline; LCL = lower confidence limits for the difference; UCL = upper confidence limits for the difference; LN = lymph node.

Table 2. Effects of buprenorphine on protective efficacy—immunologic response after immunization and homologous challenge with 5 x 10^8 cfu of *S. flexneri* 2a 2457T

	Day 7						Day 14					
Variable	BUP Mean	SAL Mean	Diff	LCL	UCL	*P*-value	BUP Mean	SAL Mean	Diff	LCL	UCL	*P*-value
Serum IgG	4.71	4.51	0.20	-1.18	1.58	0.72	5.56	5.66	-0.10	-0.58	0.38	0.60
Serum IgA	4.11	3.86	0.25	-0.48	0.98	0.40	4.36	4.36	0.00	-0.34	0.34	1.00
Spleen IgG	2.20	1.80	0.40	0.05	0.76	0.02[a]	1.56	1.57	-0.01	-0.35	0.32	0.93
Spleen IgA	2.12	1.73	0.39	0.02	0.75	0.02[a]	1.50	1.57	-0.07	-0.46	0.32	0.65
Spleen IgM	1.63	1.30	0.33	-0.06	0.72	0.06	1.24	1.32	-0.08	-0.59	0.42	0.68
LN IgG	2.22	2.27	-0.05	-0.28	0.18	0.60	1.78	1.60	0.18	-0.20	0.54	0.26
LN IgA	2.15	2.27	-0.12	-0.44	0.18	0.36	1.83	1.64	0.19	-0.06	0.45	0.08
LN IgM	1.79	1.75	0.04	-0.33	0.41	0.79	1.20	1.19	0.01	-0.30	0.33	0.90

[a]Statistical significance denoted at $P < 0.05$.
See Table 1 for key.

tion, using *Shigella flexneri* serotype 2a 2457T. However, it significantly increased the amount of ocular discharge, making assessment of the ST severity ratings more difficult and time consuming. Also, in concurrence with a previous study done in rats (38), buprenorphine significantly affected the amount of weight gain during the treatment period. During the protective efficacy study, buprenorphine had no affect on ST severity ratings, serum antibody responses, or local immune responses as measured by ASC in the cervical lymph nodes. The only statistical difference was detected on day 7 of this study, where the number of ASC in the spleen of the buprenorphine-treated guinea pigs was greater than that of the controls.

The immunomodulatory effects of select analgesics, principally the classic opioids, such as morphine, have been well-documented (39-44). These, and similar findings, are often cited as the needed justification for the non-alleviation of pain associated with use of the guinea pig keratoconjunctivitis model. However, to the authors' knowledge, the immunomodulatory effect of buprenorphine, when used in conjunction with the guinea pig keratoconjunctivitis model, had not been evaluated prior to this study. Lack of documentation of the effects of analgesics on biological variables in literature historically has been used as justification for not using them (32). However, USDA Animal Care Policy No. 11 states that analgesics must be used for potentially painful procedures, unless it has been documented that they clearly interfere with a study. Due to wide interspecies variability in dosage, absorption, biotransformation, and excretion of analgesic drugs, a protocol for the specific drug and animal species is always needed. (5, 6). Researchers practicing good science must be concerned for the well-being of the animals used in their studies, since health problems, pain, and stress may introduce unwanted variability that can confound and invalidate study results (45). In addition, unrelieved pain may have a profound impact on physiological responses, such as inhibition of the immune system (46, 47). Understanding and control of these variables allow improvement in standardization and increase in reliability of the results obtained (33).

Buprenorphine hydrochloride, a mixed agonist-antagonist opioid analgesic, was chosen for evaluation as it is the most frequently selected agent used to provide pain relief in biomedical research (48). Its relative potency and extended duration of action provide clinical and logistic advantages in comparison with most other opioids. The drug's effect on the inflammatory response of the ST was previously evaluated and was found to have no effect on the severity of disease developing during a positive ST other than the increase in ocular exudate and crusting of the eyelids, as mentioned previously. This finding was not significantly evident, however, when lower dosages were used (0.025 mg/kg) (32). In addition, buprenorphine has been reported to have no significant effects on select immunologic parameters measured in rats or mice (49, 50). A recent report also supported these findings, concluding that buprenorphine can be used in New Zealand White rabbits without compromising virulence assays (51). These references indicate that, although opioid analgesics have been found to affect specific immunologic parameters, this does not preclude their use under circumstances where the measured endpoint is not affected by their use.

Although the extent of pain relief afforded by administration of buprenorphine went undetermined in these studies, the drug's analgesic properties, including analgesiometric antinociceptive median effective dose (ED_{50}) values, have been well-characterized (52). Present results, specifically with regard to *Shigella* antigen virulence determination and the ST, concur with the aforementioned findings and will serve to add to the limited body of knowledge on the immunomodulatory properties of analgesics. Although minimal differences were detected in experiments 1 and 2, group mean splenic IgG and IgA immunoglobulin values on day 7 of the protective efficacy studies were found to reach statistical significance (IgG, $P = 0.02$; IgA, $P = 0.02$). Considering the number of variables evaluated and the lack of statistical adjustment, such as the Bonferroni method for multiple comparisons, the reported significant results may be conservative. Nevertheless, on the basis of our unadjusted findings, further evaluation of splenic immunologic response may be warranted.

As previously mentioned, the excess mucopurulent ocular discharge between test and control group animals increased the amount of time necessary for completion of the ST, requiring frequent cleaning of the eyes to avoid adherence of the eyelids. However, it had no effect on the test itself and, therefore, does not constitute sufficient justification for non-alleviation of pain. An additional consideration is the weight loss incurred in the buprenorphine-treated animals over the five-day treatment period necessary for observing development of disease. In both experiments, buprenorphine-treated animals lost 5.5 to 5.8% of the body weight observed on day 0, whereas saline-treated animals gained 4 to 5% of the day-0 body weight.

An additional concern is increased stress to the animals resulting from drug administration and frequent necessity of cleaning of the eyes. Since the ocular exudate contains virulent shigellae, and the size of the inoculum necessary to infect humans is only 100 to 200 organisms, cleaning the eyes presents an additional biosafety hazard that should only be performed with use of proper precautions. The increase in mucopurulent ocular discharge, along with the associated weight loss observed in the buprenorphine-treated animals, suggests that the effect of this analgesic may be due, to some extent, to narcosis, leading to inactivity, decreased grooming behavior, and reduction of food intake.

Results of these studies indicate that buprenorphine can be used in conjunction with the guinea pig keratoconjunctivitis model without resulting in significant immunologic effects on the parameters measured in this study. However, because of the additional observed effects of the drug, further studies using different dosing regimens or different analgesics may be warranted.

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