RESEARCH REPORT
SWINE HEALTH

Title: The adjuvant properties of *E. coli* 933D and bacterial DNA in promoting protective immunity to unrelated enteric pathogens – **NPB# 01-061**

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I. Abstract

Colonization of isolator piglets with a mild enterohemorrhage strain of *E. coli* (EHEC) results in a 2-3 fold increase in serum IgG and IgM levels compared to colonization with benign *E. coli* but does not influence serum IgA levels. Colonization with EHEC results in protection to lethal challenge with enterotoxigenic *E. coli* (ETEC) and partial protection against *Salmonella* as opposed to colonization with benign *E. coli*. We also show that colonization alone results in antibodies to fluorescein (FLU) and trinitrophenyl (TNP) even in piglets never exposed to these irrelevant antigens. The use of bacteria DNA (in the form of CpG ODN) in germfree piglets has little effect on serum Ig levels but a profound effect on the immune response to FLU and TNP although these piglets are not protected against ETEC. CpG ODN given to piglets colonized with benign *E. coli* have serum IgG levels approaching those in piglets colonized with EHEC but higher IgM and IgA levels. It is unclear as to whether they are protected against ETEC. Unexpectedly, LPS and colonization suppress the IgG anti-FLU and anti-TNP responses induced by CpG ODN administered i.p.

Our findings are relevant to the swine industry since they suggest that protection against enteric pathogens may depend on colonization with mild pathogens and that colonization stimulates innate immunity by raising the level of natural antibodies. Thus mild pathogens may serve a beneficial role in neonate survival and may have potential for providing protection in the absence of specific vaccines and antibiotics. Our data on bacterial products show they have profound immunoregulatory activity. Studies of this nature are in their infancy but in the future could greatly improve the efficacy of vaccines or colonization of the gut in reducing piglet mortality and morbidity.

II. Introduction

Enteric disease in young piglets causes morbidity and mortality in conventional and segregated early weaning that are the result of *E. coli, S. choleraesuis, S. typhimurium, S. suis, A. suis* and certain viral pathogens. While illness and death resulting from these agents may have multiple causes, the level of immunocompetence of the piglet could be an important factor. An early event in the life of the newborn is colonization of the GI tract. Colonization with benign, commensal flora is known to

provide protection from enteric pathogens, either because of the principle of ecological exclusion or because the process alteres the newborns physiology in a protective manner. Studies in laboratory animals (1-3) and farm animals (4) have demonstrated that colonization of the GI tract promotes development of the neonatal immune system and diversification of the antibody repertoire (5-7). Our laboratory is interested in defining the mechanism whereby colonization promotes immunological development in the newborn piglet and identifying the microbial agents that are required. Our efforts should be of considerable interest to the industry since they involve finding ways to stimulate protective natural or innate immunity in the newborn piglet. This could reduce or eliminate the development of conventional specific vaccines and the use of antibiotics and other costly treatments.

III. Objectives

1. Test the hypothesis that colonization with a mild enterohemorrhagic *E. coli* (EHEC) but not a benign commensal, can stimulate the development of protective immunity to enterotoxigenic *E. coli* (ETEC), Salmonella, *Streptococcus suis* and *A. suis*.
2. Test the hypothesis that bacterial products such as DNA, can also stimulate protective immunity to the enteric pathogens listed above.
3. Measure the degree to which colonization with benign *E. coli*, EHEC and the administration of bacterial products, influences antibody repertoire development at the protein and genetic level.

IV. Procedures

1. Animal studies. Isolator piglets were obtained, maintained and monitored for bacterial contamination as previously described (6). Piglets were colonized during the first week of life with 1 x 10^8 of G58-1 *E. coli,* 1 x 10^8 933D *E. coli* (EHEC) or maintained GF. In certain studies, GF piglets were given weekly intraperitoneal (i.p.) injections of 2 or 10 mg of sterile CpG ODN that has a sequence that is highly effective for stimulation of swine B-cells (kindly provided by Dr. A. Krieg, University of Iowa). Other GF piglets received LPS and MDP in addition to CpG ODN. In addition, most animals received i.p. injections of 3 mg of sterile fluorescein-conjugated hemocyanin (FLU-KLH) and TNP-Ficoll as an irrelevant antigen controls.
2. Samples collection. Prior to colonization and each week thereafter, 2.0 ml of blood was collected for recovery of B-cell RNA, plasma and differential leucocyte blood cell analysis. Weekly fecal and skin swabs were also taken from each animal and cultured for evidence of contamination and/or colonization with the inoculating strain.

Two animals in each group were sacrificed at the beginning of week 5 and the tissues listed below collected for the preparation of RNA: tonsil, parotid gland, ileal Peyers patches, mesenteric lymph node (MLN), spleen, thymus and ileum. Samples of ileum, MLNs and spleen were also collected at sacrifice for bacterial analysis (see V5 below).

3. Serological studies. The concentration of plasma/serum IgG, IgA and IgM were measured in weekly blood samples by sandwich ELISA as previously described (6, 7). Using an ELISA-based Specific Antibody Immunoassay (SpAbI), the activity of IgG, IgA and IgM (measured in ELISA units) to fluorescein (FLU) and trinotrophenol (TNP) and the various bacteria used in the research, were measured. Bacteria were immobilized in methyl glyoxal onto microtiter wells (8, 9). Data for anti-FLU and anti-TNP were also expressed as specific activity (10) for each.
4. Diversification of the heavy chain variable region repertoire. V_H and D_H usage were determined by the preparation of cDNA for each isotype followed by cloning

into the pCR-4 TOPO vector. VDJs from individual clones were then analyzed by Southern blot hybridization using V_H gene-specific probes (11).

5. Microbial culture. Samples of the tissues indicated above were aseptically collected, homogenized and cultured on blood agar both aerobically and anaerobically. The presence or absence of challenge strains was documented and contaminants, if present, were identified.

6. Pathogen challenge. All non-sacrificed animals in each group (four/group) were inoculated orally with ETEC or *Salmonella sp*. Animals were monitored for up to one week for symptoms of disease. Animals suffering severe disease symptoms were euthanized.

V. Results

A. EHEC colonization significantly effects systemic but not mucosal immunity. Figure 1 shows that colonization with the mild EHEC pathogen results in a 2-3 fold elevation of serum IgG and IgM compared to animals colonized with the benign G58-1. Interestingly, no difference in serum IgA levels were seen and in data recently published (7) no differences were seen in the degree of immunological development in the ileal Peyer patches (IPP) between colonization with the two strains.

Figure 2 shows that IgG and IgM responses to the irrelevant antigens TNP and FLU are increased in EHEC-colonized piglets although in data recently published, no differences in specific activity were seen (7).

B. Colonization alone can cause production of anti-FLU and anti-TNP in non-immunized piglets. Figure 2 also includes data from piglets that were colonized with G58-1 but never immunized i.p. with FLU-KLH and TNP-Ficoll (G58-1 IM versus G58-1 and 933D IM versus 933D). These data show that immunization is not required for the production of specific antibodies but colonization is required.

C. CpG ODN, LPS and MDP have little effect on serum Ig levels in GF piglets but CpG ODN has a major adjuvant effect on the response to both TD and TI-2 antigens. Figure 3 compares the serum Ig levels of piglets colonized with 933D, G58-1and GF piglets given weekly doses of 2 mg of CpG ODN. Very little effect on serum Ig levels in the latter group were observed. Similar results were obtained when LPS and MDP were given to GF piglets (data not shown; 12). Interesting is the profound effect of CpG ODN in GF piglets on IgG anti-FLU and anti-TNP responses (Fig. 4). While CpG ODN alone has little effect on serum Ig levels (Fig. 3) when given i.p. to piglets colonized with G58-1, serum IgG levels are increased compared to colonization with G58-1 alone whereas serum IgM and IgA levels are greatly elevated during weeks 3-5 compared to what is seen with piglets colonized with either G58-1 or 933D (Fig. 3).

D. Colonization with benign *E. coli* or LPS can significantly reduce the adjuvant effect of CpG ODN. The data presented in Figure 4 indicate that not all major bacterial products given i.p. act in a positive synergistic fashion. Furthermore, while CpG given to G58-1 colonized piglets augments their serum Ig levels (Fig. 3), either colonization or LPS can suppress the profound anti-TNP and anti-FLU response seen when CpG ODN alone was given to GF piglets (Fig. 4).

E. Colonization with the mild EHEC pathogen 933D protects against lethal challenge with ETEC and Salmonella. Figure 5 shows that piglets colonized with 933D are totally protected against a lethal challenge with ETEC (Group D). Surprisingly, these animals are somewhat semi-protected against challenge with *S. typhimurium*. Definitive data are not available on whether G58-1 colonized piglets receiving CpG ODN or CpG ODN + LPS, are also protected against challenge with ETEC.

F. Conclusions. Although frustrated by the time limits of a one-year grant for studies on animals with a 3 1/2 month gestation (as opposed to 22 days in mice), the

work generated several findings that have practical relevance to the industry. First, the effect of colonization on immunological development is not generic. The mild EHEC causes a 2-3 fold elevation of serum IgG and IgM levels and serum IgG and IgM responses to irrelevant antigens. Especially interesting is that colonization with this organism results in protective immunity to ETEC and somewhat to Salmonella. Since serum IgA or IPP development are unaffected, protection to ETEC must reside in serum IgG (or IgM) antibodies rather than in IgA-mediated mucosal immunity. Since EHEC is a pathogen, we suspect that this systemic response is the result of bacterial translocation of 933D to the peritoneum. Thus, translocation cannot be all bad, since our data imply it is necessary for protection to ETEC. Thus serum IgG antibodies to bacteria pathogens obtained by passive transfer of IgG via colostrum, should be protective for neonatal piglets. This might imply that immunization of the dam prior to parturition might be a practical treatment. Unfortunately, time and animal availability did not allow us to test for protection to *A. suis* and *S. suis*.

A second important observation was that colonization alone (Fig. 2) results in serum antibodies to TNP and FLU in animals never exposed to these antigens. Thus, colonization triggers the innate immune system and the differentiation of B-cells with anti-FLU and anti-TNP activity encoded in their genome. This observation may be a hint as to why colonization with EHEC results in protection to ETEC. Perhaps colonization or certain bacterial products that can stimulate the innate immune response may be sufficient for the generation of protective immunity in neonates.

The profound effect of CpG ODN on anti-FLU and anti-TNP responses given to GF piglets (Fig. 4) may suggest that serum responses to the antigens of bacterial pathogens are also increased. Since these responses are suppressed either by colonization with G58-1 or MDP plus LPS (Fig. 4) this observation may have little practical significance. This is supported by experiments showing that CpG treated GF piglets are not protected against challenge by ETEC (Fig. 5). Since conventional animals are exposed to both bacterial DNA and LPS, the value of synthetic DNA (CpG ODN) as an adjuvant for 3 *protective immunity*, remains to be determined.

Like all scientific studies, our work offers clues for the design of more profound experiments while generating more questions than answers. Our animal studies also generated many samples that await analysis of antibody repertoire development in the various situations described. Thus they serve as a valuable library for future research.

Citations

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Publications

Francis, D., J. E. Butler, P. Weber, J. Sun, A. Krieg and M. Sinkora. 2001. Bacterial colonization or CpG oligodeoxynucleotides are required for newborn piglets to respond to TD and TI-2 antigens. 6th International Veterinary Immunology Symposium, Uppsala, Sweden, July 16-20.

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Butler, J. E., P. Weber, M. Sinkora, D. Baker, A. Schoenherr, B. Mayer and D. Francis. 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to respond to T-dependent and type 2 T-independent antigens. J. Immunol. (In press, December).

Butler, J. E., D. Francis, P. Weber and A. Krieg. 2003. Antibody repertoire development in fetal and neonatal piglets. IX. Intestinal colonization and LPS suppresses the adjuvant effect of CpG ODN on immune responses in germfree piglets. (Submitted).

Figure Legends

Figure 1. The levels of serum IgG, IgA and IgM in GF isolator piglets and those colonized with G58-1 (Group C) or 933D (Group D). Major observation are summarized in the lower right quadrant.

Figure 2. The serum antibody response to a thymus-dependent antigen (IgG and IgM anti-FL-KLH; A, C) and the IgG and IgM response to a type 2 thymus-independent antigen (TNP-Ficoll; B, D). Arrows indicate the time of immunization and boost. Data expressed as means and standard error. The treatment groups are the same as in Figure 1.

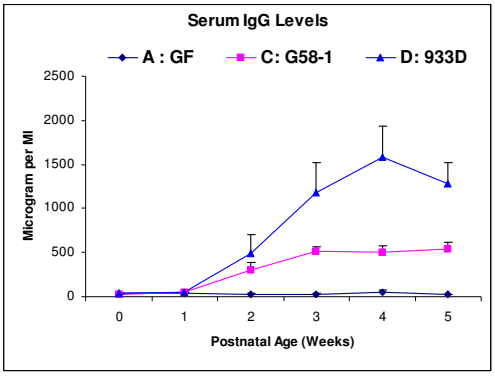
Figure 3. The influence of bacteria DNA (as CpG ODN) given i.p. to germfree (GF) piglets and to piglets colonized with G58-1 (G58-1 + CpG). Data on control GF piglets and those only colonized with G58-1 are also shown.

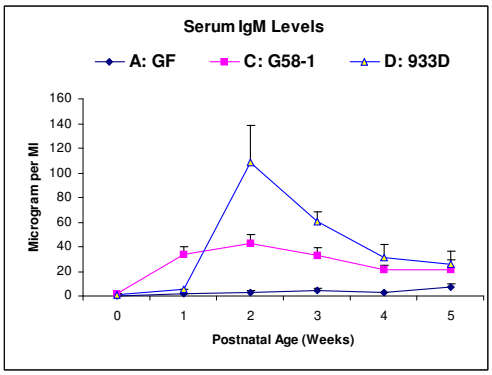
Figure 4. The immunosuppressive effect of colonization (G58-1 + CpG) or MDP + LPS on GF piglets given CpG ODN simultaneously (GF + MDP + LPS + CpG) in terms of their IgG response to fluorescein (FLU, left) and trinitrophenyl (TNP, right). The response of control animals (GF) and those receiving only CpG (GF + CpG) are also shown.

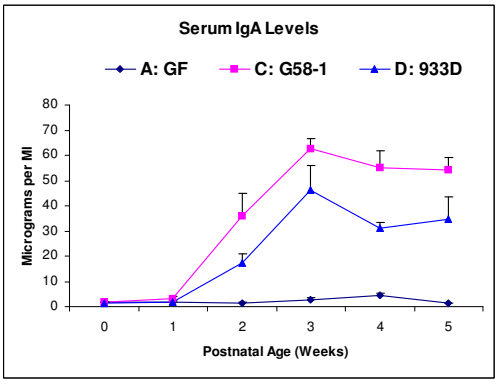
Figure 5. The effect of colonization of isolator piglets with the benign commensal G58-1 and enterohemorrhagic *E. coli* 933D and their clinical symptom index after pathogenic challenge with ETEC and *S. typhimurium*. Groups A and B are germfree controls although Group B received intraperitoneal injections of CpG ODN.

Figure 1

Colonizing Organisms differentially Effect Serum Immunoglobulin Levels







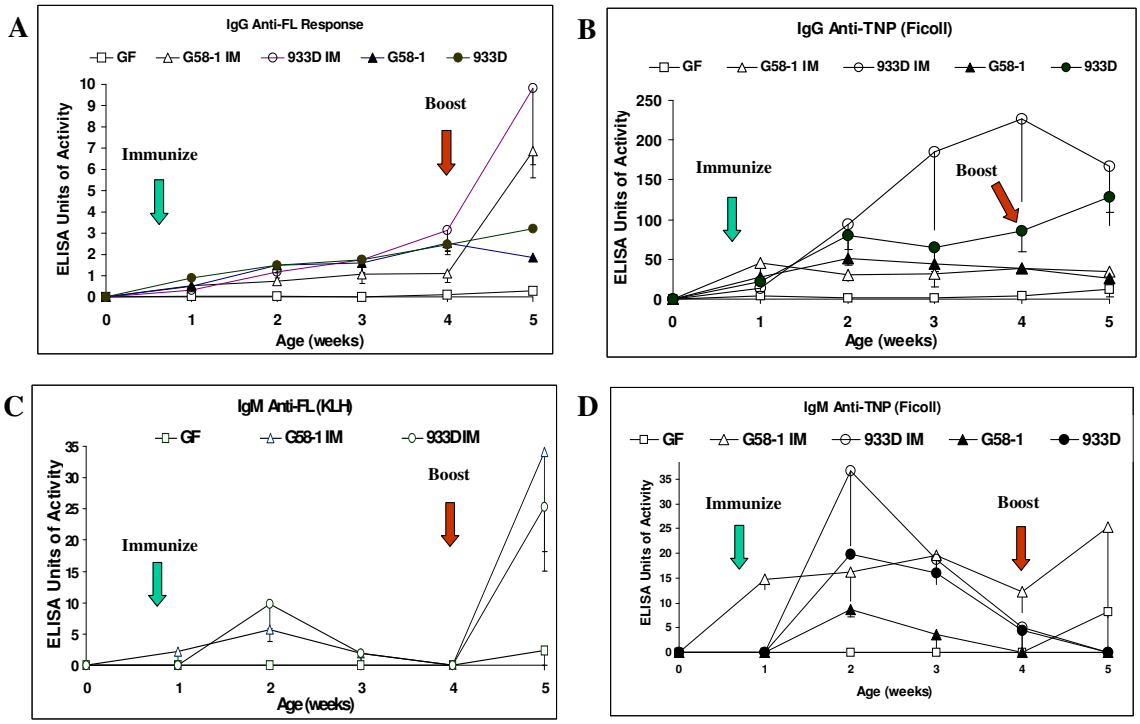
Colonization with 933 D causes a preferential increase in IgG levels

Colonization with G58-1 is equally effective as 933D in raising IgA levels

Colonization with either strain promotes a classic IgM primary response

Germfree controls have only trace levels (10-50 µg/ ml) of serum immunoglobulins

Figure 2


A
IgG Anti-FL Response
GF
G58-1 IM
933D IM
G58-1
933D
ELISA Units of Activity
Immunize
Boost
Age (weeks)
B
IgG Anti-TNP (Ficoll)
GF
G58-1 IM
933D IM
G58-1
933D
ELISA Units of Activity
Immunize
Boost
Age (weeks)
C
IgM Anti-FL (KLH)
GF
G58-1 IM
933DIM
ELISA Units of Activity
Immunize
Boost
Age (weeks)
D
IgM Anti-TNP (Ficoll)
GF
G58-1 IM
933D IM
G58-1
933D
ELISA Units of Activity
Immunize
Boost
Age (weeks)

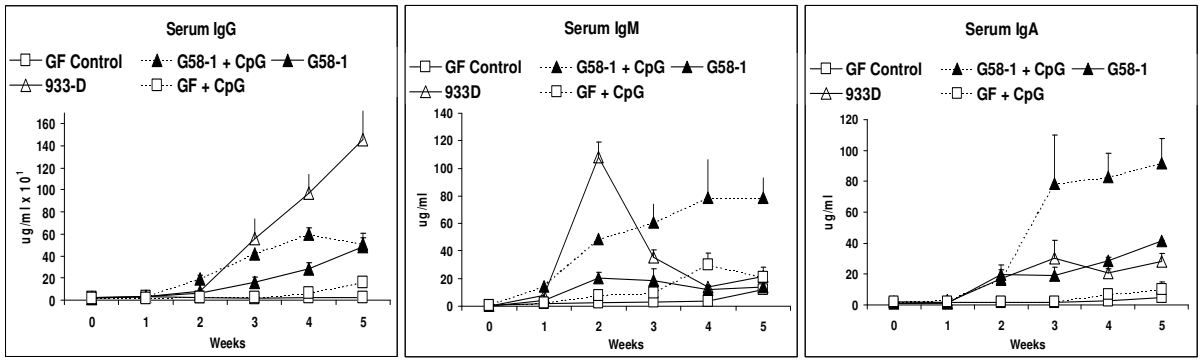


Figure 3. Bacterial DNA given to GF piglets has little effect on serum Ig levels but significantly elevates all serum Ig levels when given to piglets colonized with benign E.coli G58-1. Like CpG ODN, LPS and MDP have little effect on Ig levels in GF piglets (data not shown)

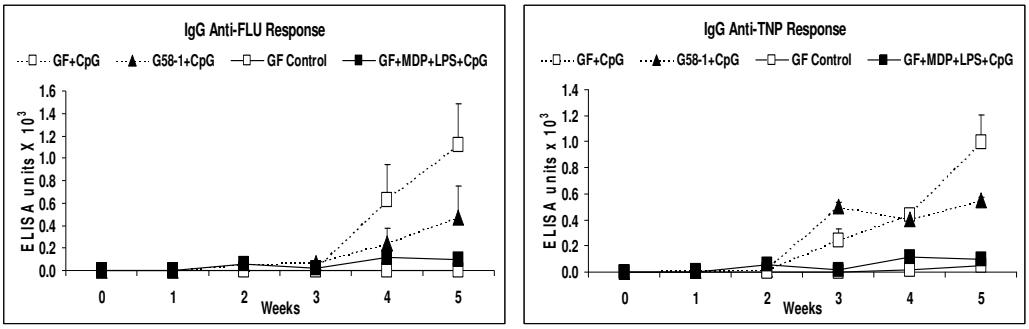
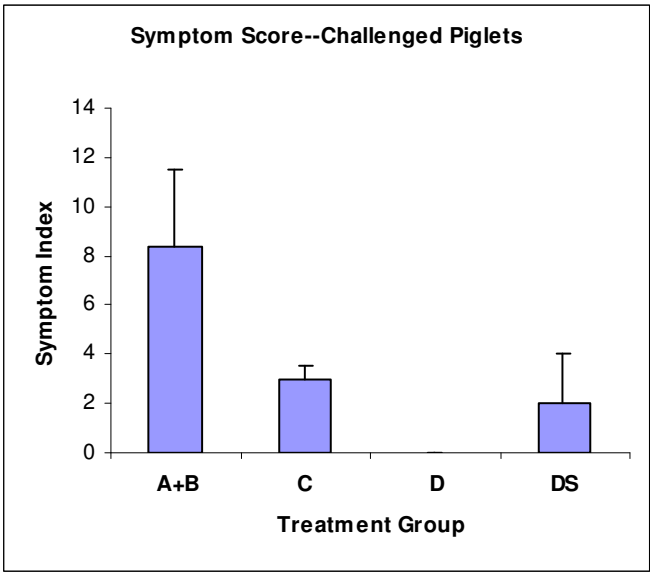


Figure 4. Colonization with benign E.coli G58-1 or LPS given i.p. suppresses the IgG anti-FLU and anti-TNP response stimulated by CpG ODN in GF piglets

Figure 5



Symptom Index: Based on degree of diarrhea, anorexia, hyperemia, CNS symptoms and death

Animal Group	Colonization	Pathogen Challenge
A & B	Germfree	*E.coli* 2134 F18
C	*E. coli* G58-1	*E. coli* 2134 F18
D	*E. coli* 933D @	*E. coli* 2134 F18
DS	*E. coli* 933D @	*S. typhimurium*

@ Derived from 0157-H7 minus shiga toxin