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DE NOVO INITIATION OF SPECIFIC CELL-MEDIATED IMMUNE RESPONSIVENESS IN CHICKENS BY TRANSFER FACTOR (SPECIFIC IMMUNITY INDUCER) OBTAINED FROM BOVINE COLOSTRUM AND MILK

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Summary. — Transfer factors (TF) were prepared from colostrum and milk of bovines previously immunized with antigens obtained from *Coccidioides immitis*, infectious bovine rhinotracheitis virus, or from the viral agents responsible for avian Newcastle disease, laryngotracheitis disease or infectious bursal disease. The ability of bovine TF to transfer specific cell-mediated immune responsiveness to a markedly xenogenic species was studied using specific pathogen free (SPF) and standard commercial (SC) chickens as model recipients. Cell-mediated immune responsiveness was documented using one or more of the following for each antigen (organism) studied: (a) an *in vitro* chicken leukocyte (heterophil) migration inhibition assay; (b) delayed-wattle reactivity; or (c) protection from clinical disease. Chicken TFs obtained from spleens of immune donors were evaluated in parallel to bovine TF's in selected comparative studies. Bovine TF also referred to as specific immunity inducer (SII), and chicken TF were found to initiate antigen-specific cell-mediated immunity *de novo* in previously non-immune SPF chickens as well as in SC chickens despite the presence of maternally acquired humoral antibody which may serve as a "barrier" to immunization of SC chickens when commercially available vaccines are administered by parenteral routes. Bovine TF's specific for laryngotracheitis virus or infectious bursal disease virus afforded protection equal to that found for commercially available vaccines. Bovine TF's action was rapid (less than a day) and of relatively long duration (at least 35 days).

Key words: avian laryngotracheitis, bovine colostrum and milk, bovine transfer factor, cell-mediated immunity, infectious bursal disease, leukocyte migration inhibition, Newcastle disease, Specific Immunity Inducer

Introduction

Previous published accounts of interspecies transfers of cell-mediated immune responsiveness (CIR) using transfer factor (TF) have focussed predominantly on gentically disparate mammalian donors and recipients

(Ascher *et al.*, 1976; Basten and Croft, 1978; Kirkpatrick *et al.*, 1983; Klesius, 1981). Over the past several years, we have been investigating interspecies transfers of CIR using animals which are markedly xenogenic; namely, bovine donors of TF and avian recipients. Our investigations have been conducted for several reasons: first, to obtain information regarding the degree of conservation of TF's structure phylogenetically; second, to assess the potential of our commercial (Amtron, Inc.) products (specific immunity inducers; SII's) that embody TF's obtained from bovines immunized with antigens of etiologic agents responsible for certain avian diseases; and third, to acquire answers to fundamental questions regarding the nature or mechanism of action of TF.

In this report, we present results from experiments which indicate that bovine TF can initiate CIR *de novo* in chickens and that bovine TF is antigen specific. In these experiments, TF's generated by immunization of bovines with antigens of *Coccidioides immitis* or with antigens of the viral agents responsible for avian Newcastle disease (ND), infectious laryngotracheitis (ILT) and infectious bursal disease (IBD) were utilized after their purification from bovine colostrum and milk (Wilson *et al.*, 1981; 1982; Wilson and Paddock; 1985). Transfer of CIR was assessed using specific pathogen free (SPF) or standard commercial (SC) chickens as model xenogenic recipients. For each antigen, one or more of the following tests served to document responsiveness: a) an *in vitro* leukocyte (heterophil) migration inhibition assay (LMI); b) delayed-wattle reactivity (DWR); or c) protection from clinical disease. Avian TF's generated to homologous antigens were evaluated in parallel to bovine TF's in selected comparative studies.

Materials and Methods

Chickens and Housing. SPF chickens of both sexes were obtained from Spafas, Inc. (Connecticut). Various strains of SC chickens (Dekalb, Black Sex-Linked, Golden Hall Cross, Cornish, and Moyer) were obtained from either Pee Dee Hatchery (South Carolina) or other independent contractors. The chickens were obtained at one day of age and housed in standard heated booder units until 28 days of age or in standard growing units beyond 28 days of age. Birds were housed, watered, and fed *ad libitum* in accordance with normal husbandry practices (U. S. Dept. of Health and Human Services, 1985). The SPF chickens had no pre-existing immunity to any of the antigens tested, whereas, the SC chickens had normal levels of maternally derived antibody at birth to ILT virus, IBD virus and ND virus.

Virus Strains and Vaccines. The following vaccines and challenge viruses were used in the experiments described herein: (a) Newcastle Disease Vaccine B1 type, B1 strain live virus (Salsbury Laboratories, Inc., Charles City, Iowa); (b) the Texas G. B. (Boney) virulent Newcastle disease virus (obtained from the U.S.D.A., Ames, Iowa); (c) Bursal Disease Vaccine, Lukert strain, live virus (Vineland Laboratories, Vineland, New Jersey); (d) Bursal disease virulent challenge virus (obtained from the U.S.D.A., Ames, Iowa; original isolate from S.A. Edgar); (e) Fowl Laryngotracheitis Vaccine, modified live virus (Salsbury Laboratories'Inc.); (f) Infectious Laryngotracheitis, virulent virus (obtained from the U.S.D.A., Ames, Iowa); (g) Bovine Rhinotracheitis – Parainfluenza-3 Vaccine, modified live virus (Nasalgen, Wellcome Animal Health Inc., Kansas City, Missouri); (h) Coccidiosis Spherule Vaccine, formalin killed then washed and resuspended in isotonic phosphate-buffered saline, Silveira strain (Naval Biomedical Research Laboratory, Naval Supply Center, Oakland, California).

Immunization of Chickens. Chickens that were used as a source of spleens to prepare immune dialyzable splenic leukocyte extract (DSLE) were first given two or three injections of com-

mercially available ILT or ND vaccine (spaced at least 14 days apart) by an ocular or subcutaneous route. The ILT vaccine contained approximately 10^3EID$_{50}$ (embryo infective doses, 50%) per dose (Salsbury Laboratories) and the ND vaccine contained approximately $10^{6.5}$ EID$_{50}$ per dose (Salsbury Laboratories). Prior to sacrificing for the preparation of immune DSLE, the chickens were confirmed to be responsive to antigens homologous to those used for injection by LMI assay. SPF chickens which were not vaccinated served as a source of spleens to prepare non-immune DSLE and were documented to be nonresponsive to ILTV or NDV antigens by LMI assay.

Chickens which were used in experiments performed to correlate antigen responsiveness in the LMI assay with *in vivo* CIR as measured by delayed-wattle reactivity or as positive controls for evaluating the effects of DSLE or bovine TF were injected once with commercially available ILT vaccine (10^3 EID$_{50}$, ocular or subcutaneous route; Salsbury Laboratories), ND vaccine ($10^{6.5}$ EID$_{50}$, ocular route; Salsbury Laboratories), IBD vaccine ($10^{4.6}$ or 10^2 EID$_{50}$, oral route; Vineland Laboratories) or *C. immitis* (0.1 mg, intramuscular route; Coccidiosis Spherual vaccine).

Preparation of Chicken Dialyzable Splenic Leukocyte Extract. Spleens from five or more chickens were used to prepare a single DSLE preparation. Leukocytes were first obtained by expressing cells from minced spleens through stainless-steel mesh. The DSLE was then prepared following procedures similar to those described earlier for the preparation of human DLE (Welch *et al.* 1976). The final concentration of reconstituted stock lyophilized dialysate was 4.0×10^9 splenic leukocyte equivalents per ml of pyrogen free water.

Preparation of Bovine TF. Proprietary experimental products which embody bovine TF as an active agent are called SII (name officially recognized by the U.S.D.A.). Several SII's were evaluated in this study; each was prepared from colostrum or milk from bovines specifically immunized with defined immunogens following procedures described previously (Wilson *et al.*, 1981; 1982; Wilson and Paddock, 1985). Prior to the preparation of a given SII from colostrum or milk the bovine donor was documented to have CIR to the immunogen used. The following SII preparations were used in the experiments described here: ILT-SII; ND-SII; IBD-SII; Infectious Bovine Rhinotracheitis-SII (IBR-S I); and *C. immitis*-SII. The activity of bovine-TF in each SII product is defined as a minimum effective dose (MED). In chickens one MED is the minimum amount of SII required to elicit CIR to a given antigen in a one to ten day old chicken. Cellular immune responsiveness is measured using the chicken LMI assay (see below).

All experimental products used in this report were required to pass rigid tests for sterility (purity) and safety as defined in the Code of Federal Regulations of the United States Government (9 CFR Sections 113.26, 113.28 and 113.36).

Intradermal Wattle Test. Delayed-wattle reactivity in each chicken was examined 14 days after a single immunization or 3 days after receiving DSLE or SII following the methods described by Giambrone *et al.* (1983) with minor modifications. Briefly, each bird was challenged by intradermal injection of one-tenth the immunization dose of immunogen into one wattle, whereas the contralateral wattle served as the control and received phosphate buffered saline only. In cases where birds were subliminally "primed" they received a challenge dose of antigen 14 days prior to receiving DSLE or SII. Reactions were considered positive when the absolute difference in thickness between wattles (antigen injected versus control) was $\geqq 0.3$ mm when measured 24 hours post-challenge (Palladino *et al.*, 1978).

Chicken Leukocyte (Heterophil) Migration Inhibition Assay. A direct "under agarose" leukocyte (heterophil) migration inhibition assay was developed to evaluate CIR in young chickens. This assay was modified from a capillary tube LMI assay described by Timms (1974) and a direct under agarose LMI assay described by Wilson *et al.* (1978; 1979). Chicken leukocytes were purified from blood obtained by cardiac puncture. The amount of antigen to be used in the LMI assay to differentiate between nonimmune and immune chickens was first established by evaluating vaccinated immune birds (with documented antibody responses), non-vaccinated, non-immune SPF chickens, and non-vaccinated SC chickens following procedures similar to those used to set-up human LMI assays (Wilson and Fudenberg, 1981). The optimal amount of each antigen is referred to as the "1.0 $\times$ concentration". Responsiveness to phytohemagglutinin (PHA) was employed as a general indicator of the potential for CIR. Responses to PHA or antigen were quantified by determining a migration index (MI) (Wilson and Fudenberg, 1981). MI values $\leqq 0.80$ were accepted as indicating significant CIR to antigen or to PHA. If a leukocyte preparation did not indicate responsiveness to PHA, all data obtained using that preparation was considered invalid and was not included in the results.

Clinical Trials. Results from clinical trials performed to determine the efficacy of LT-SII,

IBD-SII or ND-SII in preventing disease produced by virulent forms of ILTV, NDV or IBDV in chickens are presented to illustrate various points to be made in this report. The clinical trials were performed using protocols which were concordant with U.S.A.D. requirements to document the clinical efficacy of commercial vaccines (Title 9 Code of Federal Requlations of the United States Government). All challenge viruses were obtained from the U.S.D.A. (Ames, Iowa) and were administered at levels and by the routes recommended by the U.S.A.D. (10^4 EID_{50} of the Texas G.B. strain of ND virus given by intramuscular route; 10^3 EID_{50} of IBD virus given by ocular route; $10^{4.8}$ EID_{50} of LT virus given intratracheally). Additional details concerning each clinical trial are provided in the Results.

Results

Correlation of Leukocyte Migration Inhibition Assay with Vaccination and Delayed-Wattle Reactivity

Table 1 shows representative results obtained for CIR to ILTV and NDV as measured *in vitro* by LMI assay using leukocytes obtained from non--vaccinated SC chickens, non-vaccinated SPF chickens or vaccinated SC chickens. There were no significant differences in mean MI values for PHA responsiveness (5.0 μg/ml) between chicken strains or between vaccinated and non-vaccinated chickens. Non-vaccinated SC on SPF chickens had no detectable CIR to LTV or NDV, whereas, vaccinated chickens responded to both viral preparations (Table 1). We also could not find significant CIR to antigens of IBD virus, Marek's disease virus or infectious bronchitis virus in SPF chickens or non-vaccinated SC chickens regardless of the strain evaluated (data not shown). It would appear, therefore, that non-vaccinated SC chickens have no detectable CIR passively acquired from the mother even though passive transfer of antibody is known to occur.

We found the LMI assay to be specific in detecting CIR in vaccinated chickens and to correlate well with delayed-type hypersensitivity as measured by delayed-wattle reactivity (DWR). Chickens responded *in vitro* and *in vivo* only to antigens homologous to those used for vaccination (Table 2).

Table 1. Cellular immune responsivenes to infections laryngotracheitis virus or newcastle disease virus shown by a direct chicken leukocyte migration inhibition assay

Type	Antigen Concentration	Response to NDV[a] Migration Index (Mean ± SD)	Response to ILTV[a] Migration Index (Mean ± SD)
Non-Vaccinated, SC Chickens	1×	0.99 ± 0.05	0.94 ± 0.04
	1/5×	1.02 ± 0.03	0.98 ± 0.02
Non-Vaccinated, SPF Chickens	1×	1.02 ± 0.03	0.99 ± 0.01
	1/5×	0.99 ± 0.02	1.02 ± 0.02
Vaccinated SC Chickens	1×	0.72 ± 0.03	0.68 ± 0.04
	1/5×	0.88 ± 0.04	0.77 ± 0.07

[a]Response for 40 to 50 chickens.

Table 2. In vitro and in vivo cellular immune responsiveness in vaccinated and non-vaccinated chickens

Chickens Vaccinated with:	Number	Response In Vitro[a] (LMI; MI)			Response in Vivo[b] (DWR; mm × 10^2)		
		ILTV	NDV	*C. immitis*	ILTV	NDV	*C. immitis*
Coccidioides immitis	5	0.97 ± 0.60	1.02 ± 0.11	0.71 ± 0.05	N.T.	N.T.	N.T.
Infectious Laryngotracheitis Virus	11	0.64 ± 0.10	0.99 ± 0.04	1.00 ± 0.03	53 ± 8	13 ± 6	N.T.
Newcastle Disease Virus	12	0.92 ± 0.01	0.70 ± 0.03	0.95 ± 0.07	8 ± 6	70 ± 18	N.T.
Non-Vaccinated	17	0.90 ± 0.05	1.00 ± 0.02	1.03 ± 0.08	10 ± 8	15 ± 5	N.T.

[a]Response to 1× concentration of antigen.
[q]N.T. = not tested. DWR of > 30 (0.3 mm × 10^2) is considered a significant DWR.

Table 3. Transfer of cell-mediated immune responsiveness between chickens

Status of Donors of DSLE	Status of Recipients[a]	Response in Recipients After LMI ILTV	LMI *C. immitis*	DWR (mm × 10^2) ILTV	DWR (mm × 10^2) *C. immitis*
Vaccinated with ILTV but not *C. immitis*	Not Primed	0.70	1.00		
	Primed	0.72	0.90	60	21
Not Vaccinated	Not Primed	0.99	0.92	10	N.T.
	Primed	0.98	0.95	6	9

[a]Primed chickens received an intradermal injection of antigen 14 days prior to receiving DSLE. All recipients received 10^9 splenic cell equivalents of DLSE subcutaneously three days prior to evaluating their cellular immune response to ILTV or *C. immitis*.

[b]Values shown are mean migration indexes for five or more individual chickens. MI values ≤ 0.80 indicate significant cellular immune responsiveness in the leukocyte migration inhibition assay. Delayed wattle reactivity of > 0.3 mm (30×10^{-2}) indicates a significant response. N.T.: not tested.

Intraspecies Transfers of Cell-mediated Immune Responsiveness

Table 3 presents a sample of the results we have obtained from our experiments which evaluated intraspecies (chicken to chicken) transfers of CIR. We routinely found that transfers of DWR with immune DSLE could not be demonstrated using either SPF or SC recipients unless they had been previously primed using an antigen homologous to the vaccine utilized to induce immunity in DSLE donor chickens. The transfers of CIR were

Table 4. Specificity of induction of cell-mediated immunity in chickens using specific immunity inducers of bovine origin[a]

Preparation Given to Chickens	Response (MI)[b] *C. immitis*	ILTV[c]	IBDV	NDV	IBRV
1. Combination IBR-, NDV-, ILT-SII	0.90	0.70	1.01	0.75	0.79
2. IBR-SII	0.94	1.00	0.95	0.91	0.70
3. Combination *C. immitis*-, IBR-SII	0.62	0.94	1.00	0.99	0.76
4. Combination IBD-, ND-, IBR-SII	0.99	0.98	0.62	0.69	0.72
5. Combination ND-, IBR-SII	1.03	1.00	0.91	0.62	0.80
6. Combination IBD-, IBR-SII	1.00	0.90	0.65	1.01	0.78
7. Untreated Controls	0.97	0.92	1.05	0.99	1.05

Notes:

[a]Recipient SPF or SC chickens were 1 to 5 days old when given SII, 3 — 14 days old when evaluated for responsiveness. Chickens in Groups 1 to 6 received approximately 10 minimum effective doses of the indicated SII preparation by a subcutaneous or oral route.

[b]Values are Mean Migration Index (MI) for 3 to 9 experiments.

[c]Abbreviations: SII Specific Immunity Inducer; IBRV: Infectious Bovine Rhinotracheitis virus; ND: Newcastle Disease; ILT: Infectious Laryngotracheitis; IBD:Infectious Bursal Disease.

Table 5. Prevention of Newcastle disease in SPF chickens administered ND-SII after challenge[a]

Group and Treatment	Survivors Number	(Per cent)
1. Absolute Control: Not treated	1/10	(10)
2. ND-SII Preparation 24/10	9/10	(90)
3. ND-SII Preparation 24/11	8/10	(80)
4. ND-SII Preparation 24/4	8/9	(89)
5. IBR-SII Preparation 12/2	1/10	(10)

[a]SPF chickens were challenged at day 26 of life with 10^4 EID_{50} Texas GB NDV. They were treated on day 27 (24 hours later) with 15 minimum affective doses of the SII preparation noted.

specific, however, since priming of the recipients with unrelated antigen (e. g. *C. immitis*; Table 3) did not produce significant DWR after the administration of immune DSLE, nor was DSLE from non-immune chickens effective in producing DWR regardless of the status of the recipients (primed or not primed).

Subliminal priming was, however, not necessary to document significant transfers of CIR with DSLE using the LMI assay (Table 3). We have been successful in transferring CIR to both SPF and SC chickens using immune DSLE obtained from chickens vaccinated with either ILTV or NDV. In all of our experiments, the transfer of CIR were antigen specific (e. g. Table 3). We feel that the ability to demonstrate CIR in SPF chickens which received immune DSLE indicates that CIR may be initiated *de novo* (presumably by chicken TF).

Xenogenic Transfers and Specificity of Induction of Cellular Immune Responsiveness

In the course of developing SII's on an industrial scale for use in the prevention of various avian diseases, we have rigorously evaluated the antigen specificity of TF and thoroughly documented that bovine TF can initiate *de novo* CIR in previously non-immune SPF or SC chickens. In this section and the following section of this report we present results which substantiate these claims.

In Table 4 we present a summary of our results obtained using the ILMI assay to measure CIR in SPF on SC chickens which were administered SII preparations containing one or more bovine TF's. Several points can be made here: a) in each instance previously non-responsive chickens were induced to respond only to antigens which the bovine donor was immunized with; therefore, bovine TF is antigen specific; b) chickens can be induced to respond to several different antigens simultaneously provided TF's specific for each of the antigens are present in the SII preparations they are administered; c) the results obtained for SPF or SC chickens (regardless of the strain used) were essentially identical (the results shown therefore are

Table 6. Prevention of laryngotracheitis in SPF chickens administered ILT-SII either before or after challenge[a]

Group and Treatment	Chickens without clinical symptoms[b] Number	(Percent)
1. Untreated Control	5/15	(33)
2. ILT-SII Before Challenge	13/16	(81)
3. Vaccinated Control	16/19	(84)
4. ILT-SII After Challenge	7/10	(70)

[a]SPF chickens 14 days of age were treated as shown in Groups 1, 2 and 3. Thirteen days later all chickens were challenged with virulent ILTV. Two days after challenge 10 birds from Group 1 were treated with ILT-SII (Group 4).
[b]See test for definition of clinical symptoms.

a composite of those obtained using SPF and SC chickens). It would appear, therefore, that the presence of passively transferred maternal antibody does not interfere with the ability of TF to initiate CIR; d) It would also seem that bovine TF can initiate CIR *de novo*.

It is noteworthy that bovine TF, like chicken TF, was not capable of eliciting DWR in unprimed chickens, even though it routinely induced CIR as measured by the LMI assay. In primed chickens, however, bovine TF induced specific delayed-type hypersensitivity (data not shown).

Evidence from Clinical Trials for De Novo Initiation and Long Duration of Cell-Mediated Immune Responsiveness in Chickens Administered Bovine TF

We found that bovine-SII takes less than one day (hours) to initiate CIR in chickens. To substantiate this property claimed for SII (TF), we present representative results showing that ND-SII can prevent death in SPF chickens challenged 24 hours previously with the Texas-GB strain of NDV and results showing that ILT-SII can prevent morbidity or mortality in SPF chicks challenged 48 hours earlier with a virulent strain of ILTV (Tables 5 and 6).

The Newcastle disease results selected are for three identical formulations of ND-SII (sub-serials) and are taken from a study involving over 500 SPF birds. Forty-nine 26-day-old SPF chickens previously housed in the same room with SPF chickens vaccinated with the B1 Lasota stain of NDV vaccine were challenged i. m. with 10^4 EID_{50} of the Texas GB strain of NDV. The chickens were then divided into approximately equal groups and administered either ND-SII (15 MED), IBR-SII (15 MED) or nothing (untreated controls) (Table 5). The untreated, challenged SPF birds died within 72 hours (Group 1, Table 5). Three replicate formulations of ND-SII, however, prevented mortality in 80—90% of SPF birds even when administered 24 hours after challenge. In contrast, 90% of the birds treated with IBR-SII died. The failure of IBR-SII to afford significant benefit to the chickens supports a contention that ND-SII is promoting specific protection against Newcastle disease.

Table 7. Prevention of bursal disease by IBD-SII[a]

Group and Treatment (Route)	Chicken without morbidity[b] Number	(Per cent)
1. IBD-SII 5 MED (SQ)	13/16	(81)
2. IBD-SII 1 MED (SQ)	13/20	(75)
3. $10^{4.6}$ EID_{50} IBD Vaccine (Water)	14/17	(82)
4. 10^{2} EID_{50} IBD Vaccine (Water)	6/20	(30)
5. Untreated Controls	6/20	(30)

[a]Moyer chicks were treated at 1 day of age, then challenged 35 days later. N = 20 per group at day 1. A normal commercial dose of Lukert vaccine is $10^{4.6}$ EID_{50}. MED: minimum effective dose of IBD-SII. Chicks were seronegative at time of challenge (VN antibody).
[b]See text for definition of morbidity

In the clinical trial of laryngotracheitis summarized in Table 6, SPF chickens 14 days of age were either injected with 20 MED ILT-SII subcutaneously; vaccinated (1 dose modified live ILTV vaccine) or left untreated. The vaccinated chickens were kept isolated from the other chickens. Thirteen days later, all the chickens were challenged intratracheally with $10^{4.8}$ EID_{50} of virulent ILTV. Two days after challenge, 10 infected control SPF birds were treated with ILT-SII (20 MED, subcutaneously). Intratracheal challenge with virulent ILTV produces morbidity within 2 to 4 days and may be fatal in a significant number of SPF chickens (range 40—75%; Hanson, 1984). As the results in Table 6 show, ILT-SII afforded protection equal to the vaccine whether it was given thirteen days before challenge (Group 2, Table 6), or two days after challenge (Group 4, Table 6).

There are three points to note here: 1) the efficacy of our ND-SII or ILT-SII was predicted using the LMI assay, not by DWR; 2) protection was afforded by *de novo* induction of immunity by bovine TF in SPF chicken; and 3) the action of bovine TF was quick and specific.

The results for laryngotracheitis (Table 6) also illustrate that TF's effects last for at least 13 days. In the infectious bursal disease clinical trial we will review next we have substantiated that the duration of bovines TF's effects is at least 35 days in chickens. Experiments are currently in progress to determine the maximum duration of bovine TF's effects in chickens.

To evaluate the ability of IBD-SII to prevent the morbidity associated with virulent IBDV challenge, Moyer chicks one day old were treated with either: a) IBD-SII alone; b) Lukert IBD vaccine alone (orally to reduce interference by maternal antibody) or c) nothing (untreated controls). Thirty-five days later when the untreated control chickens were seronegative (virus neutralization antibodies) all the birds were challenged with a U.S.A.D. approved virulent strain of IBDV.

One MED of IBD-SII (as predicted using the chicken LMI assay) provided over a twofold reduction in the percentage of morbid chickens as compared

to the untreated control group (Group 2 versus Group 5; 65% versus 30% without morbidity). Five MED of IBD-SII provided protection equivalent to that provided by a full dose of vaccine (Group 1 versus 3; 81% versus 82%).

Discussion

In our experiments which evaluated CIR in vaccinated and non-vaccinated chickens, we found the direct agarose LMI assay to be a valid *in vitro* correlate of delayed-type hypersensitivity (as measured by DWR) in chickens. Chickens responded *in vitro* and *in vivo* only to antigens homologous to those used for vaccination (Table 2). Establishment of a good correlation between the LMI assay and DWR did require, however, careful documentation of the optimal concentration of antigen to use in both tests. This requirement has been stressed previously in connection with evaluations of CIR using human leukocytes in LMI assays (Wilson and Fudenberg, 1981). Our results are concordant with those of other investigations who also found the LMI assay to be a valid *in vitro* correlate of *in vivo* delayed-type hypersensitivity in chickens (Fauser *et al.*, 1973; Gilliland *et al.*, 1982; Zwilling *et al.*, 1972). The LMI assay is, however, in our opinion far superior to DWR for use in assessing CIR since CIR can be determined with the LMI assay in chickens as young as one day of age. The measurement of DWR can only be performed with chickens which are several weeks old.

The transfer of CIR between chickens has been evaluated previously using DWR by several investigators (Chi *et al.*, 1982; Giambrone *et al.*, 1983; Klesius *et al.*, 1977). To our knowledge, however, no one has previously reported evaluating intraspecies transfers of CIR in poultry using the LMI assay. Chi *et al.* (1982) were the first investigators to report that DWR could be transferred between chickens. In their studies, however, whole spleen cells or peripheral blood leukocytes from bursectomized donors were used to transfer DWR; DSLE or leukocyte extracts were not evaluated. Previously, Klesius *et al.* (1977) had reported unsuccessful attempts to transfer DWR between chickens regardless of whether whole blood, purified peripheral blood leukocytes or dialyzable peripheral blood leukocyte extracts from immune donor chickens were used. In these studies, Klesius *et al.* (1977) did not subliminally prime the recipients prior to giving them preparations from immune birds. Giambrone *et al.* (1983) later reported successful transfers of DWR to tuberculin, diphteria toxoid and keyhole limpet haemocyanin using DSLE obtained from splenic leukocytes of immune chickens; however, in all cases the demonstration of transfer of DWR using DSLE seemed to require subliminal priming of the recipients. In our experiments, we routinely found that transfers of DWR with immune DSLE could not be demonstrated using either SPF or SC recipients unless they had been previously primed using an antigen homologous to the vaccine utilized to induce immunity in the DSLE donor chickens (Table 3). The transfer of CIR was specific, however, since priming of the recipients with unrelated antigen did not produce significant DWR after the administration of immune DSLE, nor

was DSLE from non-immune chickens effective in producing DWR regardless of the status of the recipients (primed or not primed).

Subliminal priming was, however, not necessary to document significant transfer of CIR with DSLE using the LMI assay. We were successful in transferring CIR to both SPF and SC chickens using immune DSLE obtained from chickens vaccinated with either LTV or NDV. In all of our experiments, transfer of CIR was antigen specific (eg. Table 3). We feel that the ability to demonstrate CIR in SPF chickens which received only immune DSLE indicates that CIR may be initiated *de novo* (presumably by chicken TF).

The disparity between our results obtained *in vivo* using DWR and *in vitro* with the LMI test is not without a precedent. Croft *et al.* (1980) have previously reported that non-immune mice will demonstrate responsiveness to specific antigen by macrophage migration inhibition assay after receiving murine TF without a requirement for prior subliminal sensitization with antigen. To demonstrate delayed-type hypersensitivity *in vivo*, however, (using an ear thickness assay) the recipient mice first had to be subliminally sensitized with antigen.

To our knowledge, no one has previously published experimental evidence documenting that bovine TF can induce CIR in chickens. We have provided experimental evidence in this report which we feel substantiates that bovine TF (SII) obtained from colostrum or milk (Wilson and Paddock, 1985) can initiate antigen-specific and clinically protective CIR in previously non-immune SC or SPF chickens (Tables 4, 5, 6 and 7). Bovine SII's specific for infectious laryngotracheitis virus or infectious bursal disease virus provided protection equal to taht found for commercially available vaccines. Our finding that colostrum and milk of immunized bovines harbors TF (SII) which can induce clinically protective immunity provides evidence that for the first time an economical source of this immune modulator/potentiator has been developed.

Demonstrations of transfers of DWR with bovine SII required prior subliminal priming of the recipients with antigen. In this regard bovine SII behaved quite similar to chicken TF (DSLE). Bovine SII, like chicken TF, however, could readily be documented to transfer antigen specific CIR using the LMI assay without the necessity of first subliminally priming either SPF or SC chicken recipients with antigen (Table 3 and 4). We also found that the presence of maternally derived antibody in SC chickens had no effect on the ability of bovine SII to initiate CIR. In our Quality Assurance laboratory, we have found the LMI assay to be a valuable tool both for the measurement of TF potency and for the documentation of TF specificities in a variety of bovine SII preparations. Indeed, in all of the experiments reported herein which evaluated SII, the LMI assay was utilized to quantify the dosage (number of MED) of SII which each recipient would receive. Our results for the clinical efficacy of IBD-SII in infectious bursal disease illustrate just how well the MED predicted using the LMI assay can correlate with actual clinical efficacy. In our experiments, one predicted MED provided over a twofold reduction in the percentage of morbid chickens found in the untreated controls (Table 7).

The strongest evidence that bovine SII can induce CIR *de novo* came from experiments and clinical trials which utilized SPF chickens without pre-existing CIR as recipients (Tables 4 and 6). To our knowledge previous attempts to study *de novo* initiation of CIR using TF have not utilized SPF animals. Thus, an unequivocal answer was not forthcoming. In our experiments CIR was initiated quickly in chickens by a single administration of SII of bovine origin (as shown by our clinical trials with laryngotracheitis and Newcastle disease) and persisted for at least 35 days (Tables 5, 6 and 7). Our ability to transfer clinically protective CIR using bovine TF (SII) may also indicate that TF's structure is predominantly conserved phylogenetically.

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References

Ascher, M. S., Gottlieb, A. A., and Kirkpatrick, C. H. Eds) 1976): *Transfer Factor Basic Properties and Clinical Applications.* Academic Press, New York, 757 pp.

Basten, A. and Croft, S. 1978): Transfer factor: clinical usage and experimental studied, pp. 83—120. In: *Immunological Engineering.* MTP Press, England.

Beard, C. W. and Hanson, R. P. 1984): Newcastle disease, pp. 452—470. In: *Diseases of Poultry.* Iowa State University Press, Ames, Iowa.

Chi, D. S., Palladino, M. A., Romano, T., and Thorbecke, G. J. 1982): Transfer of delayed hypersensitivity in the chicken. *Develop. and Comp. Immunol.* **6**, 541—547.

Croft, S., Edwards, J. M., and Basten, A. 1980): Transfer of cell-mediated immunity with cell-free leukocyte extracts. II. Demonstration of antigenspecific and nonspecific components. *Cell. Immunol.* **56**, 427—439.

Fauser, I. S., Purchase, H. G., Long, P. A., Velicer, L. F., Mallman, V. H., Fauser, H. T., and Winegar, G. B. 1973): Delayed hypersensitivity and leukocyte migration inhibition in chickens with BGG or Marek's Disease Infection. *Avian Pathol.* **2**, 55—61.

Giambrone, J. J., Klesius, P. H., and Yu, M. (1983): Adoptive transfer of delayed-wattle reactivity in chickens with a dialyzable leukocyte extract containing transfer factor. *Poultry Sci.* **62**, 767—771.

Gilliland, T., Klesius, P. H. and Giambrone, J. (1982): Inhibition of chicken leukocyte migration *in vitro*: a direct agarose plate assay. *Comp. Immun. Microbiol. Infect. Dis.* **5**, 457—468.

Hanson, L. E. (1984): Laryngotracheitis, pp. 444—451. In: *Diseases of Poultry.* Iowa State University Press, Ames, Iowa.

Kirkpatrick, C. H., Burger, D. R., and Lawrence, H. S. (Eds) (1983): *Immunobiology of Transfer Factor.* Academic Press, New York, 437 pp.

Klesius, P., Johnson, W., and Kramer, T. (1977): Delayed-wattle reaction as a measure of cell-mediated immunity in the chicken. *Poultry Sci.* **56**, 249—256.

Klesius, P. H. — (1981): Modulation of cell-mediated responses with dialyzable leukocyte extract containing transfer factor. *Adv. Exper. Med. Biol.* **137**, 293—324.

Lukert, P. D. and Hitchen, S. B. (1984): Infectious bursal disease, pp 566—576. In: *Diseases of Poultry.* Iowa State University Pres, Ames, Iowa.

Palladino, M. A., Grebenau, M. D., and Thorbecke, G. J. (1978): Requirements for induction of delayed hypersensitivity in the chicken. *Devel. compar. Immunol.* **2**, 121—132.

Timms, L. (1974): Leukocyte migration inhibition as an indicator of cell-mediated immunity in chickens. *Avian Pathol.* **3**, 177—187.

United States Department of Health and Human Services. (1985): *Guide for the Care and Use of Laboratory Animals.* 83 pp.

Welch, T. M., Wilson, G. B., and Fudenberg, H. H. (1976): Human transfer factor in guinea pigs: further studies, pp 399—405. In: *Transfer Factor Basic Properties and Clinical Applications.* Academic Press, New York.

Wilson, G. B., Fudenberg, H. H., and Bahm, V. J. (1978): Distinct components in dialyzable leukocyte extracts have specific and nonspecific effects on cellular immunity as shown by leukocyte migration inhibition. *Trans. Assoc. Am. Phys.* **91**, 294–332.

Wilson, G. B., Fudenberg, H. H., and Horsmanheimo, M. (1979): Effects of dialyzable leukocyte extracts with transfer factor activity on leukocyte migration *in vitro* I. Antigen-dependent inhibition and antigen-independent inhibition and enhancement of migration. *J. lab. clin. Med.* **93**, 800–818.

Wilson, G. B. and Fudenberg, H. H. (1981): Leukocyte migration inhibition as a method for assaying transfer factor activities. *Lymphokines* **4**, 107–173.

Wilson, G. B., Paddock, G. V., and Fudenberg, H. H. (1981): Effects of dialyzable leukocyte extracts with transfer factor activity on leukocyte migration *in vitro*. V. Antigen-specific lymphocyte responsiveness can be initiated by two structurally distinct polyribonucleopeptides. *Thymus* **2**, 257–276.

Wilson, G. B., Paddock, G. V., and Fudenberg, H. H. (1982): Bovine "transfer factor": An oligoribonucleopeptide which initiates antigen-specific lymphocyte responsiveness. *Thymus* **4**, 335–350.

Wilson, G. B. and Paddock, G. V. (1985): Process for obtaining transfer factor from colostrum. South African Patent No. 84/9156 Granted July 31, 1985.

Zwilling, B. S., Barrett, J. T., and Breitenbach, R. P. (1972): Avian delayed-type hypersensitivity adaption of the migration-inhibition assay. *Cell. Immunol.* **4**, 20–28.